EXPRESSION OF CONNEXIN 43 (Cx43) IN ORAL SUBMUCOUS FIBROSIS AND IN ITS MALIGNANT TRANSFORMATION TO ORAL SQUAMOUS CELL CARCINOMA

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

MAY 2019

CERTIFICATE

This is to certify that this dissertation titled "**EXPRESSION OF CONNEXIN 43 (Cx43) IN ORAL SUBMUCOUS FIBROSIS AND IN ITS MALIGNANT TRANSFORMATION TO ORAL SQUAMOUS CELL CARCINOMA**" is a bonafide dissertation performed by **Dr. R.N. MUGUNDAN** under our guidance during his postgraduate study period of 2016 - 2019.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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I hereby declare that this dissertation titled "**EXPRESSION OF CONNEXIN 43 (Cx43) IN ORAL SUBMUCOUS FIBROSIS AND IN ITS MALIGNANT TRANSFORMATION TO ORAL SQUAMOUS CELL CARCINOMA**" is a bonafide and genuine research work carried out by me under the guidance of **Prof. Dr. K. Ranganathan, M.D.S., MS (Ohio)., PhD.**, Professor and Head and **Prof. Dr. Umadevi K. Rao, M.D.S.**, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

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ENDORSEMENT BY THE HEAD OF THE DEPARTMENT AND HEAD OF THE INSTITUTION

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Acknowledgement

ACKNOWLEDGEMENT

*I bow in gratitude to the **Almighty and my paternal and maternal grandparents** for all their showers of blessings.*

*My heartfelt gratitude to my teacher **Dr. K . Ranganathan** , MDS., MS (Ohio), Ph.D . , Professor and Head of Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for his constant support and encouragement throughout my course. He has always been a source of inspiration to perform better not only in academics but also in life. Thank you so much sir.*

*I extend my sincere gratitude to Professor, **Dr. Uma Devi. K. Rao**, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for her constant guidance, support and her advice in completion of my work . I want to take this opportunity to acknowledge her for the care she takes towards us to do everything perfectly in curricular activities. Thank you so much ma'am .*

*I earnestly thank Professor, **Dr. Elizabeth Joshua** , Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for her encouragement and concern in helping me to complete this study. She has always been a source of valuable advices both in academics and life. Thank you so much Ma'am.*

I extend my heartfelt special word of thanks to Professor, ***Dr. T . Rooban*** *, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for his guidance, encouragement, concern and continuous support, in the hour of need and for his valuable advices thus helping me to complete this study. Thank you so much Sir.*

My sincere thanks to the Principal ***Dr. N. S . Azhagarasan*** *and Chairman* ***Mr. Kanakaraj*** *, Ragas Dental College and Hospital for their permission to use the facilities of the institution .*

I extend my sincere thanks to Readers ***Dr. N. Lavanya*** *and* ***Dr. C . Lavanya,*** *Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for their continuous encouragement and support.*

I extend my sincere thanks to Senior lecturers ***Dr. R Sudharsan, Dr. Kavitha, Dr. Sruthi, Dr. Joseph,*** *Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for their help throughout my project.*

I am very grateful to our Geneticist and Lab Manager ***Mrs. Kavitha Wilson****, for her advices to carry out my study successfully.*

I extend my sincere thanks to our Lab Technician, ***Mr. Rajan****, Department of Oral and Maxillofacial Pathology and Attender* ***Mrs. Laila****, Ragas Dental College and Hospital for their constant help in completion of my study. I also thank* ***Mrs. Hemalatha,*** *the biostatistician, for helping me with the statistical analysis of my study.*

I acknowledge gratefully the help of my batchmates, ***Anitha J, Dhanaswathii.T, Janani S.R, Sruti Murali*** and ***Ruth Vijitha*** *for helping me complete this project.*

I acknowledge gratefully the help of all my ***Seniors*** *and* ***Juniors*** *for helping me complete this project.*

I acknowledge gratefully the help of my friends ***H. Sunil, J. Naveen, S.Raghu*** *and* ***S. Sivaraman*** *for their help in completing this project.*

I thank my parents ***Mr. R.Narendran*** *and* ***Mrs. N.Nithya*** *my brother* ***Mr . R.N. Dheepak Anand****, who stood by me through all the situations I faced.*

CONTENTS

Introduction

Head and neck cancers are the 11th most common malignancy worldwide[1]. Oral squamous cell carcinoma (OSCC) accounts for almost 95% of the malignant neoplasms which occur in the head and neck region.[2] OSCC can occur in various parts of the oral cavity; the tongue is the most frequently involved site followed by the buccal mucosa, gingiva and the palatal regions. The tumor has a high mortality and a poor overall survival rate due to its invasiveness and metastatic capacity. Since many of the lesions are asymptomatic, there is a delay in their diagnosis. Majority of the OSCCs are usually preceded by asymptomatic conditions that are called Oral Potentially Malignant Disorders (OPMD) by World Health Organization (WHO). This term was adopted since it had been found that not all of the OPMDs progressed to malignancy.[3]

Among the OPMDs, Oral submucous fibrosis (OSF) is a predominant condition of South East Asia, where it is associated with the habit of areca nut chewing. OSF has a high prevalence in the Indian sub-continent. It is usually seen in males in the age range of 20-40 years with the habit of areca nut chewing**.** The prevalence of OSF has increased over the years from 8.3% to 16.2%.[4]

The main etiological agent for OSF is chewing of areca nut with or without tobacco products. The International Association of Research on Cancer (IARC) has classified areca nut as a Type – I (Substances that are known to be carcinogenic to human beings).[5] Areca nut alkaloids such as arecoline, arecaidine, guacoline and guavacine cause disruption in the equilibrium of collagen homeostasis and upregulation of profibrotic cytokines such as Transforming growth factor- β (TGF-β). TGF-β belongs to a family of pro-fibrotic cytokine plays a role in the normal healing of wounds.

The first case of malignant transformation in OSF was reported by JC Paymaster in 1956.[6] The malignant transformation rate of OSF was reported to be around 7.6% over a 17 year period.[7] In OSF cases with dysplasia, the malignant transformation rate was 4.84% and in those OSF cases which did not exhibit dysplasia the malignant transformation was 3.72%.[8] The transformation of OSF to OSCC in another study was found to be 4.5% over a period of 14 years.[9]

Advanced stages of OSF shows dysplastic features in the epithelium characterized by loss of cellular cohesion.[10] The cellular cohesion maintains the continuity of the epithelium and keeps it intact. Oral epithelial dysplasias are more commonly associated with tobacco habits. They carry a high risk for malignant transformation. The loss of cellular cohesion is one of the architectural features of epithelial dysplasia, according to WHO criteria. Many studies have been carried out in cervical and mammary dysplasias and cell junctions. Cellular junctions comprise of tight junctions, adherens junctions and gap junctions. Gap junctions are transmembrane proteins that connect the cytoplasm of the neighboring epithelial cells by Gap junction intercellular communication channel (GJIC) through which various growth factors and secondary messengers are channeled.[11]

Connexins are specialized gap junction proteins expressed extensively throughout the tissues of the body. It is necessary for the normal functioning of epithelium and maintenance of cellular homeostasis.[12] Connexin 43 (Cx43) is the most commonly expressed isoform. It is known that the loss of gap junction leads to the loss of cellular cohesion which promotes tumor progression and invasiveness. This could potentially play a part in the malignant transformation. Expression of connexin has been studied in fibrotic diseases such as liver cirrhosis, pulmonary fibrosis, systemic sclerosis and renal fibrosis. The altered expression of this Cx43

protein has been studied in various fibrotic diseases but the association of Cx43 with OPMDs such as epithelial dysplasia and oral submucous fibrosis has not been reported in the literature. This study was aimed to study the expression of Cx43 levels in OSF, epithelial dysplasia and in OSF cases where malignant transformation had occurred.

Aims and Objectives

HYPOTHESIS:-

There is no difference in the expression of Cx43 among OSF, Epithelial dysplasia and cases where OSF had undergone malignant transformation.

AIM:-

To evaluate the expression of Connexin 43 (Cx43), in formalin fixed paraffin embedded tissues of oral submucous fibrosis, epithelial dysplasia and OSF that had undergone malignant transformation to oral squamous cell carcinoma

OBJECTIVES:-

- To study the expression of Cx43in formalin fixed paraffin embedded tissues of Oral submucous fibrosis by IHC
- To study the expression of Cx43in formalin fixed paraffin embedded tissues of epithelial dysplasia by IHC
- To study the expression of Cx43 in in formalin fixed paraffin embedded tissues of OSF with OSCC by IHC.
- To study the expression of Cx43 in formalin fixed paraffin embedded normal epithelial tissue by IHC
- To compare the expression of Cx43 among the study groups

Materials and Methods

STUDY GROUPS:-

- Group - I:Normal oral mucosa obtained with the patients consent during third molar extraction(n=6)
- Group - II: Clinically and histopathologically diagnosed cases of oral submucous fibrosis (OSF)(n=14)
- Group- III: Clinically and histopathologically diagnosed cases of Epithelial Dysplasia (n=12)
- Group-IV: Clinically and histopathologically diagnosed cases of OSCC with the history of OSF (n=7)

SELECTION CRITERIA:-

- Group I–Normal mucosa obtained during removal of impacted third molar in the outpatient department of Oral and Maxillofacial Surgery department.
- Group II–The clinical criteria for selection of OSF patients were difficulty in opening the mouth, burning sensation of the mouth, palpable vertical fibrous bands in the oral mucosa. Histopathological criteria for OSF cases were epithelial atrophy, increased deposition of collagen in the oral mucosa,reduced vascularity, reduced number of inflammatory cells, and sub-epithelial hyalinization.OSF was histopathologically graded by criteria given by Pindborg and Sirsat[13]
- Group III - Clinically and histopathologically confirmed cases of Epithelial dysplasia which were graded histologically as mild, moderate and severe
- Group IV- Clinically and histopathologically confirmed cases of OSCC with a history of OSF.

INSTITUTIONAL REVIEW BOARD:-

This study was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai.(Annexure I)

STUDY SETTING:-

Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital.

METHODOLOGY:-

- Archival tissue samples of oral submucous fibrosis, epithelial dysplasia and oral squamous cell carcinoma with history of OSF accessed from Department of Oral Pathology, Ragas Dental College and Hospital.
- Demographic and pertinent details such as patient's age, gender, past medical and dental history, history of drugs and trauma were obtained from records for OSF cases, OSCC arising in OSF cases and normal cases.
- In group I, incisional biopsy were taken when patients were undergoing minor surgery for extraction of impacted teeth for normal oral mucosa after obtaining patient consent.
- The tissue biopsied were rinsed and immediately transferred to 10% buffered formalin. After adequate fixation, tissues were embedded in paraffin.
- From the paraffin embedded blocks, 5 micron thick sections were cut and used for routine hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining.
- Positive control for Cx43 was goat heart tissue sections.

Immunohistochemistry was performed by:-

ANTIBODIES USED:-

Primary antibody –Anti-Cx43 rabbit monoclonal antibody, Dilution: 1:100 CAT NO- 71-0700 (*ThermoFisherScientific*™)

Secondary antibody-Poly Excel-HRP Micro polymer, IHC Detection Kit, CAT NO- 36000 (*ThermoFisherScientific*™)

PROCEDURE

The slides were dewaxed in xylene and rehydrated through grades of alcohol to water. The sections on the slides were flooded with Harris's hematoxylin for 5 minutes. The slides were washed in running tap water for 5 minutes. The slides were differentiated in 1% acid alcohol for 5 minutes. The slides were washed well in running tap water for 5 minutes. The tissue sections on the slides were then stained in eosin for 30 seconds. The slides were washed in running tap water for 1 minute. The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope.

IMMUNOHISTOCHEMICAL STAINING:-

Armamentarium:-

1) Microtome
2) Autoclave
3) Hot air oven
4) Slide warmer
5) Coplin jars

6) Measuring jar
7) Weighing machine
8) Poly L-Lysine coated slides
9) Slide box
10) Aluminium foil
11) Micro-pipettes
12) Toothed forceps
13) Electronic timer
14) Beakers
15) Rectangular steel tray with glass rods
16) Sterile gauze
17) Cover slips
18) Light microscope (LM)

REAGENTS USED

1) Poly-L-Lysine
2) 1 N sodium hydroxide
3) 1 N Hydrochloric acid
4) 0.01 M sodium citrate buffer (pH 6.0)
5) Phosphate buffered Saline (PBS)
6) 3% Hydrogen peroxide
7) Distilled water
8) Hematoxylin
9) Absolute alcohol (Isopropyl alcohol)
10) Xylene

PREPARATION OF PARAFFIN SECTIONS

After the slides were dried, tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the Poly-L-Lysine coated slides from the tissue float bath such that two tissue bits come on to each slide with a gap in between. One of the tissue sections towards the frosted end of the slide was labelled negative to which negative serum, the secondary and the chromogen were added and the tissue section away from the frosted side is the positive to which the primary antibody, secondary antibody and chromogen were added.

IHC PROCEDURE

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Circles were drawn using a diamond marker around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. To expose target proteins, antigen retrieval was performed using 10mM sodium citrate (pH 6.0), microwaved for 8-15 min. Following antigen retrieval, tissues were blocked for endogenous activity in 3% H_2O_2-methanol for 15 min at room temperature, washed with PBS, and then coated with Connexin 43 monoclonal antibody (*ThermoFisherScientific*^{TM} 138300) diluted in 3% PBS at a dilution of 1:100 overnight at 4°C. Tissues were washed in PBS and detection was performed using anstreptavidin-horseradish peroxidase (HRP)-conjugated secondary antibody followed by colorimetric detection using a 3,3-diaminobenzidine tetrahydrochloride (DAB) kit. Tissues were counterstained with

hematoxylin and dehydrated with ethanol and xylene. The Slides were mounted with DPX and observed under light microscope.

POSITIVE AND NEGATIVE CONTROL:

Section of goat heart tissue that was positive for Cx43 was used as positive control. Negative control sections were processed by omitting primary antibody.

IHC PROCEDURE:

1. Poly-L Lysine coated slides with 2 paraffin embedded tissue placed in warming table

2. Placed in xylene twice (5 minutes each)

3. Placed in 100% isopropyl alchohol (5 minutes)

4. Placed in 90% isopropyl alcohol (5 minutes)

5. Placed in 70% isopropyl alcohol (5 minutes)

6. Washed in distilled water (2 minutes each)

7. Keep in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval

8. Placed in 3% hydrogen peroxide (7 minutes)

9. Washed with PBS buffer (2-3 minutes)

10. Primary antibody Cx43 (1:100) was added and incubated at 4°C overnight

11. Washed in PBS buffer (2-3 minutes)

12. Poly excel target binder reagent added and incubated (12 minutes)

13. Washed in PBS buffer (2-3 minutes)

14. Poly excel HRP secondary antibody added and incubated (12 minutes)

15. Washed slides in PBS buffer (2-3 minutes)

16. DAB added and incubated (3 minutes)

17. Washed in PBS thrice (2-3 minutes)

18. Stained with Harris Hematoxylin (20 seconds)

19. Washed in tap water

20. Placed in 70% alcohol (1 minute)

21. Placed in 100% alcohol (1 minute)

22. Placed in xylene (1 dip)

23. Slides to be mounted using DPX

24. Slides to be observed under the LM and graded.

EVALUATION OF H & E SECTION/S:

- The H&E stained sections were thoroughly examined. Oral squamous cell carcinomas were graded as well differentiated, moderately differentiated and poorly differentiated.
- Epithelial dysplasia were graded as mild, moderate and severe.
- Oral submucous fibrosis was graded histologically by criteria given by ***Sirsat and Pindborg***[13].
- Corresponding sections as examined by H & E were stained by IHC to detect Cx43 expression.

- The positive control was a section of goat heart tissue that was previously shown to be positive for Cx43.
- Cx43 expression was evaluated as brown membranous staining in the epithelial cells.
- The cells were then assessed for staining intensity.

INTENSITY OF STAINING

The staining intensity was analysed in the study groups. Each case was graded as (-) nil or absence of stain, (+) **mild**, (++) **moderate** and (+++) **intense** staining, based on the intensity of staining taken up by the tissue as observed by two blinded observers independently with respect to positive control.

TISSUE LOCALIZATION:

Epithelial cells that exhibited brown membranous staining were counted as positive for expression of Cx43. The sections were initially scanned at low power. For sections that showed heterogeneous staining, the predominant pattern was taken into account for scoring.

CELLULAR LOCALIZATION OF STAIN:

Cx43 expression was seen as brown membranous or cytoplasmic staining. The stained slides were screened, examined systematically for Cx43 expression in membrane, cytoplasm and walls of the epithelial cell.

STATISTICAL TEST USED: The staining intensity between each groups was evaluated using Chi-squared test. For inter-observer reliability, kappa statistics was done ($p<0.01$). Data analysis to be done using *SPSS software version 21*.

Review of Literature

In 1952, Schwartz had five women from Kenya reported with stiffening and blanching of the oral mucosa, the palate andthe fauces, which was called "atrophiaidiopathica (tropica) mucosae oris". Joshi in 1953 described the condition as Oral Submucous Fibrosis (OSF).Later in 1966, OSF was defined by Pindborg and Sirsat as an"Insidious, chronic disease affecting any part of the oral cavity and sometimes pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxtaepithelial inflammatory reaction followed by fibro elastic change of the lamina propria, with epithelial atrophy leading to stiffness of oral mucosa and causing trismus and inability to eat". [13]

EPIDEMIOLOGY:-

OSF is prevalent in Southeast Asia and in the Indian Sub-continent. It is now reported worldwide due to migration of the population and the widespread use of areca nut due to commercialized preparations of areca nut. The prevalence of OSF in Southeast Asian countries like china was 1.0 - 3.03%[14] and in Vietnam was 13% [15]An epidemiological survey conducted in Taiwan by ***Shun Fa Yang et al*** that spanned over 17 years, found the prevalence of OSF increased from 8.3% in 1996 to 16.6% in 2013[16].In India, the prevalence has increased from 0.03% to 6.42% over a period of 6 years [83]and more than 5 million cases of OSF were reported from India [25]

In Southern India, the prevalence of OSF in Kerala was 2.01% and in Tamil Nadu it was 8%[84.] The gender predilection was0.2 to 2.3 % in males and 1.2–4.57 % in females. ***Ranganathan et al.,*** in their study had observed that OSF had frequently been associated with the habit of areca nut chewing with tobacco,

pan masala chewing and males had a higher prevalence[19] The malignant transformation of OSF to oral squamous cell carcinoma (OSCC) was observed by JC Paymaster in 1956 [6] It was designated as one among the Oral potentially malignant disorders (OPMD), since not all of the diagnosed OSF cases progressed to OSCC. ***Murti et al in 1986*** reported a malignant transformation rate of 4.5% to 7.6% two years later[84.] It has been suggested that longer follow up period may show a higher rate of malignant transformation.

ETIOPATHOGENESIS:-

OSF was strongly associated with the habit of areca nut chewing. The main etiological agent in areca nut is their alkaloids arecoline, a major areca nut alkaloid, whose active metabolite is arecoline N-oxide. It has been reported to have cytotoxic and genotoxic properties based on which the International Association of Research on Cancer (IARC) has classified areca nut as atype – I human environmental carcinogen[20,5] The cytotoxicity and genotoxicity of areca nut alkaloids was studied in an experiment that involved cultured human buccal epithelial cells with areca nut alkaloids - arecaline, arecaidine, guavacine, quavcolineand N-nitrosamine compounds such as N-nitrosoguvacoline. They had caused DNA strandbreaks and decreased cell survival proving their cytotoxic and genotoxic property [21]

The areca nut/betel quidfibers and their alkaloids serve as a constant source of irritation to the oral mucosa resulting in micro-trauma at the site of placement. The micro-traumaallows the diffusion of the areca nut alkaloids into

the mucosa. This results in the production of reactive oxygen species (ROS) and inflammatory

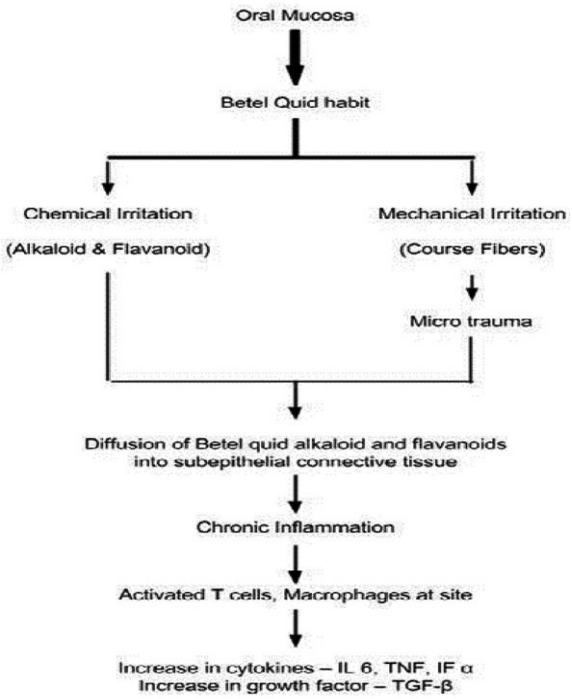


FIGURE 1: ROLE OF ARECA NUT IN INITIATION OF ORAL SUBMUCOUS FIBROSIS

cytokines such as IL-6, TNF- α, TGF- β and IFN α.(Figure 1)Alkaloids and flavonoids of the areca nut are arecoline, arecaidine, guvacine and guvacoline. Arecoline is the highly active alkaloid that causes cholinergic and anti-muscarinic effects by binding to the GABA receptors in the brain, leading to psycho-stimulativeeffects, Arecaidine has been known to stimulatecollagen production by buccal fibroblasts[22]. Areca nut flavonoidsincluding tannins and catechins promote a non-soluble, highly stable collagen structure by inhibiting collagenase activity. As OSF progresses, the type 3 collagen is completely replaced by type 1 collagen which are more resistant to degradation [23]

Role of Copper in OSF

Copper content present in areca nut is involved in the pathogenesis of OSF. The enzyme lysyl oxidase is dependent on copper for its activation. It is responsible for the increased and highly stable cross-linking of the collagen by promoting hydroxylation of the collagen fibres. ***Trivedy et al 2001*** conducted a study by culturing human fibroblasts with copper chloride and had found that copper had influenced the collagen production by the fibroblasts and the amount of collagen production was dose dependent. The same author had also studied the copper levels in the tissue of OSF, serum and urine of the individuals who had chewed areca nut for 5-10 min and it was found to be raised, concluding that copper was an initiator of OSF, which is responsible for the hydroxylation of the collagen fibres. [24]

Inflammatory cytokines and OSF:

Pro-inflammatory cytokines are produced during inflammatory reactions by macrophages. They regulate fibroblast proliferation, migration and extra cellular matrix synthesis and reorganization. The balance between the cytokines plays a significant role in the initiation and progression of fibrotic disease. ***M. F. Haque et al., 2000*** studied the production of cytokines such as IL-1β, IL-6, IL-8, TNF-α and IFN- γ from the peripheral blood mononuclear cells (PBMC) of OSF patients through flow cytometry and compared them with genetically-related relatives, who were control subjects. There was an increase in the levels of IL-1β, IL-6, IL-8 and TNF-α. These elevated levels of cytokines also had induced fibrosis in animal models [25]. Areca nut extract also helps in the production of prostaglandins (PGE) and cyclooxygenase 2 (COX-2) in oral keratinocytes and

maintains the chronic inflammatory condition seen in OSF by activating the Nf-κB pathway.

Role of TGF- β in OSF

Transforming growth factor - β (TGF- β), a pro-fibrotic growth factor, is involved in fibrosis and extracellular matrix remodeling. There are various isoforms of TGF-β. TGF- β1 was expressed in epithelial, hematopoietic, and connective tissue cells, while TGF- β2 was expressed in epithelial, neuronal cells and TGF- β3 primarily in mesenchymal cells[27]. TGF-βcauses the deposition of extra cellular matrix (ECM) by increasing the collagen synthesis and decreases its degradation by activating tissue inhibitor of matrix metalloproteinase (TIMP).The immunohistochemical expression of TGF-β was observed in 70 tissue sections of OSF in a study done by ***VV Kamath et al., 2015.*** Both the isoforms of TGF- β1 and TGF- β2 were found to be expressed in the tissues of OSF. When comparedto the control group, there was an increased expression of TGF- β1 in OSF tissues compared to TGF-β2.Connective Tissue Growth Factor (CTGF), pro-fibrotic mediator is associated with cell proliferation, adhesion, angiogenesis and tissue fibrosis in a pathologic state. ***Yi-Ting Deng et al., 2009*** studied the immunohistochemical expression of CTGF in 20 tissue sections of OSF. They concluded a strong expression of CTGF staining in the endothelium and fibroblasts and concluded that it had a high fibrogenic activity than TGF- β and synergistically may induce a strong fibrotic response.[39]

Extracellular matrix remodelling and OSF

Extracellular matrix (ECM) is composed of a network of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins. These glycoproteins bind with each other and provide a scaffold of support for the organ. Dynamic remodeling of the ECM is critical for normal development and wound healing. In the event of inflammation, when ECM remodeling gets disrupted or uncontrolled, it contributes to fibrotic diseases and malignancy.The extracellular matrix (ECM) remodeling with OSF progression was analyzed by ***Hiroko Utsunomiya et al 2005*** who studied the expression of ECM proteins such as tenascin, perlecan, fibronectin, collagen type III in 40 tissue sections of OSF through immunohistochemistry and in-situ hybridization. They found that there wasan altered expression of ECM proteins in various stages of OSF, with the most advanced stages of OSF being characterized predominantly by type I collagen fibres, which are normally resistant to degradation.[23]

Role of αvβ6 in OSF

αvβ6, an integrin protein that is upregulated in inflammatory and pathologic conditions, plays a role in fibrosis and in malignancy. Elevated levels of αvβ6 activates TGF-β, which causes myofibroblast differentiation producing type-1 collagen fibers. ***KA Moutasim et al in 2011*** tested the hypothesis if levels of αvβ6 was increased in OSF and αvβ6 expression was dependent on TGF-β for myofibroblast trans-differentiation. The level of αvβ6 expression was tested in 41 tissue sections of OSF and 14 tissues of fibrous hyperplasia. Oral keratinocyte cell line that had expressed high levels of αvβ6 was developed along with normal cell

lines that were used as a control. The results showed that there was high staining of αvβ6 in tissue sections of OSF, as inhibition of TGF-β1 showed decreased myofibroblast transformation signifying that αvβ6 was dependent of TGF-β1. Oral keratinocytes induced with areca nut alkaloid also showed an upregulation of αvβ6 integrin [30]

Myofibroblasts and OSF

Myofibroblasts are contractile fibroblasts that express α smooth muscle actin (αSMA). They are responsible for the tissue repair, cell motility, proliferation, differentiation, apoptosis, morphogenesis, tissue repair and are involved in fibrotic pathoses involving liver, lung, and kidney. Trans-differentiation of the fibroblasts to the myofibroblasts is a crucial and early event in tumorigenesis, mediated by the growth factors and TGF-β expressed by the tumor cells. Myofibroblasts secrete numerous growth factors and inflammatory mediators that stimulate epithelial cell proliferation. Assessment of α-SMA was studied in 70 tissue samples of OSF of which 35 were diagnosed histologically as an early stage and 35 were advanced by ***PV Angadi et al in 2011*** through immunohistochemistry. They found that there was a high expression of α-SMA in cases of advanced OSF when compared to controls which showed no expression.[31]

CLINICAL FEATURES OF ORAL SUBMUCOUS FIBROSIS:-

OSF is characterized by formation of palpable fibrotic bands of the oral mucosa resulting in reduced mouth opening. Stomatopyrosis with intolerance to consuming spicy food, blisters and ulcerations, dryness of the mouth followed by

vesicles, ulcers andmucosal pigmentation are commonly seen in early stages. As the condition advances, fibrosis progresses. The severity of the disease can be assessed by the severe limitation in mouth opening.In the advanced stages, there is restriction of tongue movement and shrinkage of the uvula. The faucial pillars become thick, short, and extremely hard. Leukoplakia is a common finding along with OSF, among patients with the habit of chewing tobacco products along with areca nut. [13]

HISTOPATHOLOGY OF ORAL SUBMUCOUS FIBROSIS:

Histopathological features of oral submucous fibrosis vary according to the stages of the lesion. In early stages, it is characterized by atrophic oral epithelium. The connective tissue exhibits juxta epithelial hyalinization and thickened collagen bundles. Other findings include intercellular edema, constricted blood vessels and mild inflammatory cell infiltration. ***Sirsat*** and ***Pindborg*** gave histologic criteria that divides OSF into four stages such as early stage, very early stage, moderately advanced stage and advanced stage.[13.]In advanced stages, epithelial atrophy, dense collagen, hyalinization seen extending into the submucosal tissues were seen with lack of inflammatory cell infiltrate. In advanced cases of OSF, changes in epithelial dysplastic features may be observed and it may contribute to the malignant transformation. ***Jayasooriya et al*** studied the relationship thickness in OSF in 107 cases of histologically confirmed cases of OSF and stated that the features of epithelial dysplasia becomes more prominent as the condition of the OSF progresses, thereby increasing the possibility of malignant transformation.

OSF as an Oral Potentially malignant disorder:-

OSF is an oral potentially malignant disorders (OPMD)[33] The malignant transformation of OSF into oral squamous cell carcinoma (OSCC) was first reported by Paymaster in 1956. ***Wang YY et al.,*** studied the prevalence rate of dysplasia that were associated with OSF. OSF that exhibited dysplastic features had a malignant transformation rate of 4.84% while those with no dysplastic changes showed a malignant transformation rate of 3.72% [4.] In India, the highest rate of malignant transformation of OSF was 7.6%**.** ***P Chaturvedi et al*** had suggested that OSCC arising in the background of OSF had a better prognosis than OSCC that were habit associated[36.] Various biomarkers have been studied for their suggested role in the malignant transformation of OSF such as the apoptotic markers studied by ***Ranganathan et al,*** angiogenic molecules by ***Chaudhary et al*** and reactive oxygen species by ***Uikey et al*** in OSF.

Role of Hypoxia in malignant transformation of OSF:

Hypoxia was found to play a role in malignant transformation of OSF. It was suggested that reduced blood supply allowed the accumulation of carcinogens in epithelium.***WM Tilakaratne, et al 2008*** studied 48 cases of OSF and 17 cases of epithelial dysplasiathrough immunohistochemistry and RT-PCR. They found a positive expression of Hypoxia inducible factor- 1 α (HIF-1α) in the tissues of OSF and at the mRNA level in epithelial dysplasia. In another study carried out by ***Minal Chaudhary et al in 2015***, HIF-1α expression was studied in 20 cases of OSF, OSCC and OSF associated with OSCC. The staining intensity and mean vascular density (MVD) were noted. They described the increase in the staining of HIF-1α from OSF to OSCC and the highest MVD was seen in the samples of

OSCC. They concluded that the increase in HIF-1α and MVD may play a role in the malignant transformation of OSF.[35]

Senescence associated fibroblast and OSF:

Senescent fibroblasts secrete growth factors, cytokines and degradative enzymes, which alter the tissue microenvironments and affect epithelial cells.Fibroblasts produce senescence-associated secretory phenotype (SASP) fibroblasts in response to constant DNA damage produced by ROS. A cell culture study by ***Hassona Y et al*** involving premalignant cells lines, has shown that SASP fibroblasts produce epithelial–mesenchyme transitional events by downregulating epithelial protein markers such as β-catenin, E-cadherin and cytokeratin and upregulating the expression of mesenchymal characteristics such as vimentin, N-cadherin.SASPs are known to produce high levels of TGF-β and MMP-2[39] When associated with dysplasia, the senescent cells in OSF may contribute to the development and progression to OSCC in this disease.

Cytokeratin Profiles:

Cytokeratins (CK) which are the intermediate filaments of the epithelium form the cytoskeleton of the epithelial cells.Keratinocytes in oral epithelium are always matched in basic and acidic pairs (type I and type II) and are site specific.The expression of these cytokeratins remain stable through the malignant transformation and is a reliable marker for studying malignant differentiation [40].The aberrant expression of cytokeratin has been demonstrated in various oral premalignant lesions [42] and in OSCC [41].The Cytokeratin expression in OSF was assessed through immunohistochemistry by ***Ranganathan K et al., 2006*** in 50

tissue samples of OSF and it was concluded that there was an increase in the expression of cytokeratin 8 (CK8), Pancytokeratin (panCK) and high molecular weight cytokeratin (HMWCK) from normal controls to OSF and OSCC and concluded that these changes in cytokeratin maybe used as a surrogate marker for malignant transformation [42]

Genomic Instability in OSF and OSCC

Genomic instability can be assessed through Loss of heterozygosity (LOH). LOH refers to the loss of genomic material that involves the normal copy of an allele. These regions contain oncogenes and tumour suppressor genes for normal function and maintenance. Most of the head and neck squamous cell carcinomas (HNSCC) involve the chromosomal regions such a 3p, 4q, 8p, 9q, 11q, 13q and 17p (Uma Devi k Rao 2018) Event of LOH in OSF was studied by ***MT Teh et al 2007*** through blood DNA samples of 15 OSF patients through Single nucleotide polymorphism (SNP) microarray mapping assay. They discovered 23 hotspot loci in 47-53% of the OSF samples and involved the chromosomes 3p, 6q, 9q, 12p, 13q and 20p.13q which was found to be lost in 90% of HNSCC cases, was also involved in the OSF. These LOH regions may play a role in the malignant transformation of OSF [43]

Epithelial Mesenchymal Transition

Epithelial Mesenchymal transition (EMT) describes the event in which a terminally differentiated epithelial cell acquires mesenchymal traits such as elongated, spindled shaped fibroblast like cells. This is also followed by a loss in

epithelial markers including E-cadherin, cytokeratin, zonula occludens-1 (ZO-1) and gain in mesenchymal markers such as vimentin, α- SMA and fibroblast-specific protein-1 (FSP-1) which promoted motility of the cell.The inflammatory mediators produced in response to injury cause EMT, which could lead to fibrosis.The chronic inflammation seen in OSF promotes EMT events, involving the keratinocytes.The loss of E-cadherin along with β-catenin from the membrane might have led the cytoplasmic β-catenin to regulate thecanonical Wnt signaling pathway and the molecular determinants of epithelial polarity which have tumorigenesis enhancing impact through EMT[44]

OPMDs – Epithelial dysplasia

In India, 60–80% of patients present with advanced disease stages of OSCC due to delay in the diagnosis when compared to 40% in developed countries. These conditions are usually preceded by premalignant lesions that carry a high risk of malignant transformation. Among them, leukoplakia and OSF are predominant in the Indian sub-continent due to tobacco and betel-quid usage.

Epithelial Dysplasia

Among the OPMDs, leukoplakia is the most commonly encountered lesion carrying a high risk of malignant transformation. The lesion is frequently associated with the habit of tobacco smoking in the form of cigarettes and tobacco chewing. They clinically appear as a white patch involving the floor of the mouth, buccal mucosa and the tongue and it cannot be characterized as any other lesion. They have a prevalence rate of 2% with a malignant transformation rate of 8 to 24%. Based on the presence and extent of the dysplastic features, the malignant

risk [78] could be assessed, though not all of the dysplasia present could transform to malignancy. WHO grades epithelial dysplasia into three categories: mild, moderate and severe. Mild dysplasia is characterized by architectural and cytological changes that are confined to the lower one-third of the epithelium, while in moderate and severe dysplasia, the changes are seen extending into the middle third and upto the upper third of the epithelium, respectively.When the entire epithelial thickness is involved, it is labelled as carcinoma in-situ. Recently, in WHO 2017 classification of Oral dysplasia, loss of cellular cohesion has been accepted as an architectural feature in grading dysplasia[3]. The loss of intercellular communication is frequently involved in premalignant conditions and this intercellular communication is facilitated by gap junctions. Many studies have reported the loss of gap junction in dysplasia that had occurred elsewhere in the human body such as lung, cervix.

Oral squamous cell carcinoma (OSCC) results from the chronic inflammation and multistep accumulation of genetic and epigenetic changes. OSCC has a high propensity for local invasion and cervical lymph node dissemination[45].This results in a reduced overall survival rate. Therefore, earlier detection and treatment of OPMD such as epithelial dysplasia and OSF might inhibit and prevent the malignant transformation.Various panel of biomarkers have been studied in OSCC including expression of angiogenic markers, apoptotic markers, cell senescence markers, cytokeratin profiles, genetic susceptibility,tumor suppressor genes, oncogenes, cell proliferation markers and cell adhesion molecules to predict the malignant transformation in OSF (Figure 2)[98].***Chaturvedi and Sarode et al*** studied OSF cases that were associated with a history of OSCC and found that there was an increased overall survival rate in

patients with OSF-OSCC and they had a better rate of prognosis by a better tumor differentiation and decreased chances of nodal metastases. Loss of cellular communication occurs in severe dysplastic lesions and in tumor invasion and metastasis. The cellular communication is maintained by junctions of the epithelial cells. Among them Gap junctions contribute to a significant part in maintaining the cellular architecture.

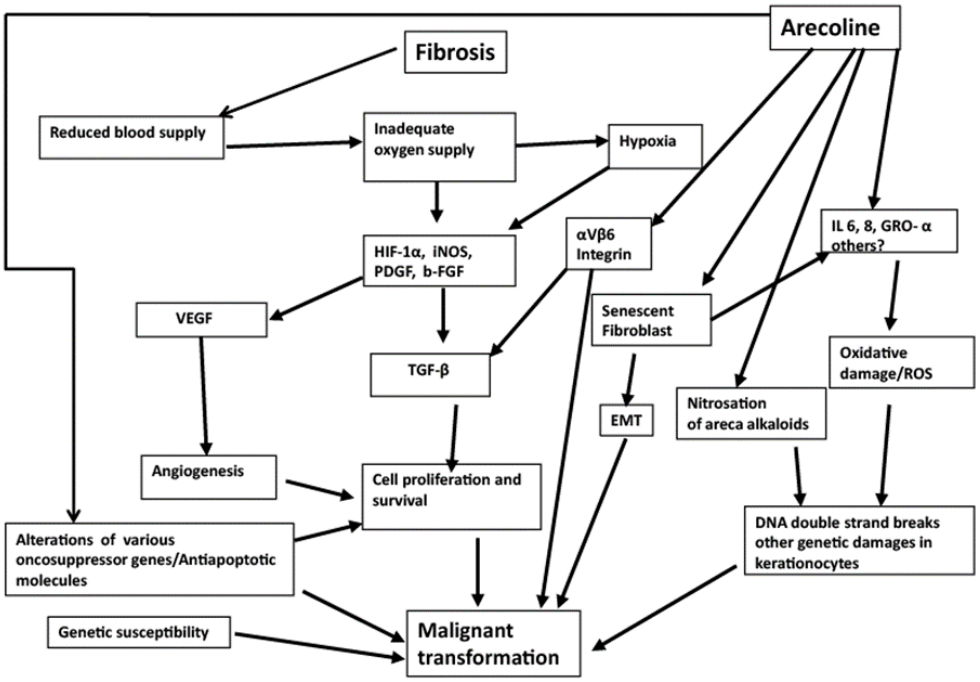


FIGURE 2. FLOWCHART SUMMARIZING FACTORS IMPLICATED IN THE MALIGNANT TRANSFORMATION OF OSF

GAP JUNCTIONS AND CONNEXIN:

Normal cell to cell communication and homeostasis is mediated through gap junctions which connects the cytoplasm of the neighboring cells to each other.The gap junctions are formed by transmembrane integral membrane proteins called Connexins (Cx).They are defined as 'Clusters of a few to hundreds of tightly packed intercellular channels that, in the simplest assessment, function to allow small molecules to be directly exchanged between adjoining cells'. [11].The

connexins form gap junction intercellular communication channel (GJIC). These channels allows the exchange of metabolites, ions, growth factors and secondary messengers between the adjacent cells.It also maintains tissue homoeostasis, cell growth control and development[48]

The connexins are synthesized in the rough endoplasmic reticulum (rER) of the golgi apparatus and oligomerizes similar to a membrane protein to form an individual connexon. These individual connexons are then transported and docked at the plasma membrane into a helical structure, where they are termed as connexin. These connexin molecules connect with the cytoplasm of the adjacent cell thereby allowing the passage of secondary messengers, ions, growth factors.[49] The connexin molecules have a narrow gap diameter of 2-4 nm. Multiple isoforms of connexins are expressed in different cell types throughout the body exhibiting a characteristic tissue and cellular distribution, signifying their functional differentiation. In humans, 21 isoforms of connexin have been identified[50]. Among them, connexin43 (Cx43) is found to be expressed in tissues of various organs.

Morphology of Connexin:-

Connexins are highly regulated integral trans-membrane proteins that consist of 2 extracellular loops, a cytoplasmic loop and cytoplasmic N- and C-terminal (Figure 3). The C-terminal domain varies in its length and provide sites for protein-protein interaction [51].Connexins are named after their prefix- as Cx and by their molecular weight as kilodalton (kD) from their primary amino acid sequence. For example, connexin43 is 43 Kd[52]

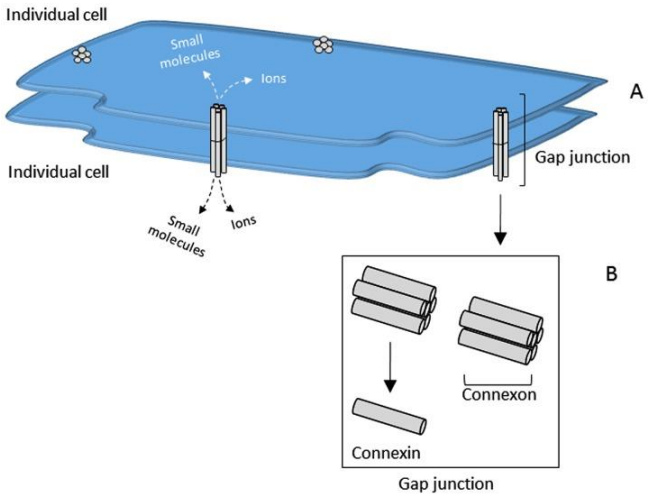


FIGURE 3: MORPHOLOGY OF CONNEXIN

Connexin expression and gap junction communication are both regulated during the cell cycle. Connexin influences cell cycle by allowing the flow of growth factors and extracellular messenger that influence the expression of cell cycle regulatory proteins such as cyclin dependent kinases (CDK) and Cyclin inhibitory proteins (CIP)[53]. Reduced expression of connexins and Gap Junctional Intercellular Communication (GJIC) has been shown in many tumor types and have been recognized as tumor suppressors. Clinical studies show that deficient or abnormal connexins are frequently found in tumor tissues and cell lines, such as breast cancer, prostate cancer, lung cancers[54] Cellular dissociation results in cell-cell recognition impairment. Progressive loss of connexin has been observed to occur in advancing tumor stages that promote the invasiveness of the tumor. Aberrant GJIC levels were found to be due to lack of expression and cytoplasmic localization of the protein.

Cx43

The connexin 43 gene, or gap junction associated protein 1(*GJA1),* is located at human chromosome 6q22-q23. Cx43 is the most widely expressed connexin isotype, found in tissues such as cardiomyocytes, keratinocytes, astrocytes, endothelial cells and smooth-muscle cells.[55]Cx43 has been extensively studied in tumors involving the breast, prostate, live r, lung and the brain as a surrogate marker for detecting early malignancy. Expression of connexin can be studied by analyzing the cellular localization and their expression level [56].In cancerous or chronic inflammatory conditions, phosphorylation and subcellular localization of Cx43 is considered to be an early event in carcinogenesis.

The involvement of GJIC in malignancy was first demonstrated by Lowenstein and Kanno who had found a disruption in the electrical coupling between the cancerous liver cells due to a loss in GJIC [57].This decrease in connexin level has been suspected to be an early event in malignancy. Depending on the staging and the type of cancer, connexin can either act as a tumor suppressor or oncogene.[58]Based on this finding, connexin localization and both upregulation and downregulation is suspected to have a role in the prognostic outcome of the disease[55]

ROLE OF CONNEXIN IN CARCINOMA:-

The levels of Cx43 expression and their role in dysplastic cells was studied by ***TJ. King et al in 2000*** through immunohistochemistry and cell culture in 10 tissue samples of cervical dysplasia. They had found that Cx43 was significantly downregulated and to analyze the effect of this down regulation, they cultured

HeLa cells and studied the conduction of Cx43. It had revealed neoplastic progression of the dysplastic cells and loss of Cx43 influenced this process [59]

Immunohistochemical analysis and genetic mutational analysis of Cx43 and Cx32 in 29 samples of colorectal carcinoma was carried out by ***MV Dubina et al in 2002.*** Their results suggested an aberrant cytoplasmic localization and connexin related disruption of GJIC during tumor invasion and metastasis. Cx43 gene was found to be mutated in the cases of colonic carcinoma and wasfound to be responsible for the advanced stage of the disease progression [60]

A study by ***Kańczuga-Koda L et al., in 2003*** analyzed the expression of Cx43 in 40 tissue samples of benign mammary dysplasias that consisted of papillomas, adenosis, cysts, intraductal and atypical hyperplasia and 25 tissue samples of normal mammary gland through immunohistochemistry. They had found that there was an altered expression (both of cytoplasmic and membranous expression) of Cx43 in the groups of cysts, adenosis, papillomas and decreased expression of Cx43 in the premalignant case of atypical ductal hyperplasia when compared to the normal mammary tissue where the Cx43 expression was seen intramembranous. The authors then concluded that loss of Cx43 was due to an early event in carcinogenesis.[61]

Tatsuya Tanaka et al in 2006 assessed the prognostic influence of Cx43 expression in 79 resected oesophageal cancer specimens with tumor grading and lymphatic invasion through immunohistochemistry. They found that 62 samples showed high positivity for Cx43 expression. They had also showed a shortened overall survival rate during post-operative follow up. This suggested a poor

prognosis when compared to the tissues that had a mild Cx43 expression. Based on this finding, they concluded that Cx43 could be used as an independent prognostic marker in esophageal carcinoma [62]

Can-Xia Xu et al in 2008 studied the expression of Cx43 and Cx32 in tissue sections of 33 cases of chronic superficial gastritis (CSG) with Heliobacter pylori (HP) infection and 88 cases of precancerous conditions and 70 cases of gastric cancer through immunohistochemistry. They had found that expression of both Cx32 and Cx43 were reduced in cases of gastric cancer associated with HP infections when compared to CSGs [63].

Tang et al in 2011studied the expression of Cx43 and E-cadherin in 92 samples of Gastric tumors and their associated lymph node metastases through immunohistochemistry. They found that there was decreased expression of Cx43 and E-cadherin in gastric tumors, whereas the metastatic lymph node showed a high cytoplasmic expression of both Cx43 and E-cadherin. They had stated that reduction in the connexin channels might influence the invasiveness of the tumor[64]

Immunohistochemical analysis of Cx43 in 348 tissue samples of urothelial carcinoma was studied by ***Poyet C et al., in* 2015**. They concluded that high expression of Cx43 staining was seen in the tissues as the stages of cancer progressed. This was a finding in contrast to the other findings found in the literature. The role of Connexin in tumours and in cancer progression varies due to cellular heterogeneity of the analysed tumours and the complex multilevel

process of tumorigenesis and progression. However, more reports suggest that Cx43 is overexpressed in some solid tumours and is involved in late metastatic steps. This could be demonstrated for breast cancer, melanoma and oral squamous cell carcinoma. [65]

Erdem and Çelik in 2017 studied the expression of Cx43 in 79 tissue samples of cervical intraepithelial neoplasia (CIL) that were graded between 1, 2 and 3 through immunohistochemistry. Normal cervical epithelium was not included in the study as controls due to their lack of availability. Endocervical glands present in the dysplastic tissues served as an internal control. They found no expression of Cx43 among the dysplastic tissues but a mild expression was observed in the endocervical glands. They concluded that the lack of expression in the dysplastic tissues was due to loss of gap junction protein Cx43, secondary to the premalignant changes.[66]

Hagemann et al in 2012 studied the expression of Cx43 in 37 tissue specimens of cervical dysplasia through immunohistochemistry and spectral imaging. They had observed the mild expression of Cx43 in clinically normal tissue adjacent to the dysplasia, followed by aberrant cytoplasmic expression in high grade squamous intraepithelial lesions (HSIL) and low staining of Cx43 in clinically normal mucosa. They concluded that the level of Cx43 expression can influence the stage of cervical intraepithelial neoplasia [67]

CONNEXIN 43 AND ORAL CANCER:

Cx43 expression in tongue carcinogenesis was first carried out by ***Juan Xia et al in 2009*** who had induced squamous cell carcinoma in rat tongue by 4-

nitroquinoline-1-oxide. 48 tissue specimens with visible lesions on the rat tongue were taken and the staining of Cx43 was observed through immunohistochemistry. There was a lack of expression in the basal cell layer andan intense Cx43 expression in the cell membrane of spinous and the granular layer. The intensity of the Cx43 expression had gradually decreased in cases of oral cancer compared to the controls. They concluded that downregulation of Cx43 wasa crucial event in oral carcinogenesis [68]

Expression of Cx43 in the OSCC induced in rat tongue by 4-nitroquinoline-1-oxide (4NQO) was studied by ***Feng Y et al in 2013*** through immunohistochemistry in tissue samples of epithelial dysplasia (mild, moderate and severe) and OSCC of the rat tongue. There was positive expression of Cx43 in the membrane of epithelial cells in stratum spinosum, stratum granulosum and a lack of expression in the basal layer, cornified layer of the normal mucosa. In the dysplastic tissues, there was a progressive decrease in the expression of Cx43 from cases of mild, moderate and severe dysplasia followed by a weak expression in OSCC. They concluded that a decrease in the expression of Cx43 is an early feature of oral carcinogenesis [69]

Feng Y et al in 2013 studied the expression of Cytokeratin 19 (CK 19) and Cx43 and their correlation through immunohistochemistry in tissue samples of epithelial dysplasia (mild, moderate and severe) and OSCC induced in rat tongue by 4-nitroquinoline-1-oxide(4NQO). They had found that there was positive expression of CK19, localized in the basal layer of normal lingual mucosa. Increased expression of CK19 was found in the suprabasal layers of mild,

moderate and severe dysplasia, In OSCC, there was a high intensity of CK19 expression. Cx43 was expressed in the stratum spinosum and stratum granulosum in normal mucosa. In dysplasia and OSCC, their expression had been reduced significantly. The expression of CK19 and Cx43 had a negative correlation and thus was concluded that the combined expression of these markers might be used in the early diagnosis of OSCC [70]

Brockmeyer et al., (2014)studied thelocalization pattern of Cx26, Cx43 and Cx45 in 35 tissue samples of OSCC along with normal oral mucosa and lymph node metastases through RT-PCR and immunohistochemistry. There was lack of Cx26 expression in normal oral mucosa but there was positive expression in the cytoplasm of neoplastic cells.The membrane expression had decreased with increasing keratinization seen in OSCC. This keratinization leads to a reduction in the cell membrane, resulting in sub-cellular localization of Cx43 following its subsequent degradation leading to an increase in the cytoplasmic expression in OSCC.[71]

The studied was continued by ***Brockmeyer P et al in 2014*** where the membranous expression of Cx43 and its prognostic value was assessed through immunohistochemistry. They had observed a high membranous Cx43 expression in samples of OSCC and lymph node metastasis which was associated with poor prognosis and reduced overall survival period. A mild membranous expression of Cx43 was also seen in clinically normal mucosa adjacent to the tumor. They concluded their study stating that high membranous expression of Cx43 was an independent prognostic marker of the tumor, which was associated with poor prognosis [72]

An immunohistochemical study by***Lidia Puzzo et al 2016*** analyzed the expression of Cx43 in 87 tissues of untreated resected specimens of laryngeal squamous cell carcinoma. They had found a mild expression of Cx43 in well differentiated and moderately differentiated carcinoma. There was no expression of Cx43 in poorly differentiated samples. The moderately differentiated carcinomas with lymph node metastasis showed aberrant Cx43 expression that was associated with decreased overall survival period during follow up and showed poor prognosis. They concluded that carcinomas which did not exhibit Cx43 expression had a better prognosis than a carcinoma having an aberrant Cx43 expression that involves metastasis and poor prognosis[73]

Results

SAMPLE CHARACTERISTICS:-

The study population comprised of 39 cases from the archival specimens of department of oral and maxillofacial pathology. The study population was comprised of four groups. Group I (n = 6) was comprised of Normal oral mucosa obtained during the extraction of third molars. Group II (n = 14) comprised of Oral submucous fibrosis samples, Group III consisted of Epithelial dysplasia (n =12) that were associated with habits and Group IV comprised of OSCC associated with OSF (n = 7). All the tissue samples were analyzed for Cx43 immunostaining

Distribution of age among the study groups (Table I & Graph I):-

The age among the study groups were divided into 3 categories: < 25 years, 25-50 years and > 50 years. In group I, 1(16.7%) case was in the age group < 25 years and 5(83.3%) the cases were between the age group 25-50 years. Group II had 7(50%) cases between the age 25-50 years of age and 7(50%) cases were in the age group >50 years. Group III had 6(50%) cases within 25-50 years and 6(50%) of cases in the age group >50 years. Group IV had 2(28.6%) cases within 25-50 years and 5(71.4%) of cases > 50 years. The difference in age distribution between the groups was not significant (p=0.081).

Distribution of gender among the study groups (Table II & Graph II):-

In group I, 4(66.7%) cases were males and 2(33.3) % were females. In group II, 10(71.4%) of cases were males and 4(28.6%) cases were females. While group

III had 11(91.7%) were males and 1(8.3%) female, group IV had 6(85.7%) was males and 1(14.3%) of females. The difference in gender distribution between the groups was not significant (p=0.490).

Distribution of habits among the study groups (Table III & Graph III):-

The habits among the study groups were divided into six categories based on their habit as those who chewed areca nut, those who chewed tobacco, those who chewed areca nut and tobacco, those who only smoked, those who smoked with tobacco and areca nut chewing and those without habits. In group I, all 6(100%) cases had no habits. In group II, 9(64.3%) chewed areca nut, 5(35.7%) had the habit of chewing areca nut and tobacco. In group III, 7(58.3%) had the habit of chewing tobacco, 1(8.3%) had the habit of chewing areca nut and tobacco, while 4(33.3%) had the habit of smoking. In group IV, 4(57.1%) had the habit of chewing tobacco and arecanut while 1(14.3%) had the habit of smoking and areca nut chewing and 2(28.6%) had the habit of smoking along with tobacco and areca nut chewing. The distribution of habits between the study groups was statistically significant **(p=0.000).**

Distribution of site of the lesion among the study group (Table IV & Graph IV):-

In group I, 3(50%) of the cases were from buccal mucosa and 3(50%) of cases were from the alveolar mucosa. In group II, all 14(100%) of the cases were from the buccal mucosa. In group III, all 12(100%) of the cases were from buccal

mucosa and in group IV, 3(42.9%) of the cases were from buccal mucosa, 1(14.3%) from alveolar mucosa, 1(14.3%) from palate and 2(28.6%) cases from the lateral border of the tongue. The difference in distribution of the site of the lesion was statistically significant between the groups **(p=0.001*).**

Distribution of Cx43 staining among the study groups (Table V & Graph V):-

All the tissue sections were analyzed for immune-histochemical staining of Cx43. Among them, in group I, 5(83.3%) cases had Cx43 expression and 1(16.7%) did not express Cx43. In group II, 13(92.9%) cases had expressed Cx43 staining while 1(7.1%) showed no expression. In group III, 8(66.7%) had Cx43 expression, while 4(33.3%) had no expression of Cx43. In group IV, 6(85.7%) had positive Cx43 expression, whereas 1(14.3%) had no expression.The difference in Cx43 expression between the groups was not significant (p=0.375).

STAINING INTENSITY:-

The Cx43 staining intensity was analyzed in the study groups and graded as (-) = Negative or absence of staining, (+) = Mild staining, (++) = Moderate staining, and (+++) = Intense staining.

INTEROBSERVER VARIATION IN Cx43 STAINING INTENSITY:-

The kappa value for inter observer variation is 0.8

Comparison of Cx43 staining of basal layer among the study group (Table VI& Graph VI):-

In group I, all of the cases 6(100%) showed no Cx43 expression. In group 2, 10(64.3%) showed no expression of Cx43, 3(21.4%) had mild expression for Cx43 and 1(7.1%) had moderate staining for Cx43. In group III and group IV, all 12(100%) and 7(100%) cases were negative for Cx43. (p=0.241).

Comparison of Cx43 staining of suprabasal layer among the study group (Table VII& Graph VII):-

In group I, 1(16.7%) case had no expression of Cx43 and 5(83.3%) cases had mild staining for Cx43. In group II, 2(14.3%) were negative for Cx43, 7(50%) case had mild expression for Cx43 and 5(35.7%) cases had moderate staining for Cx43. In group 3, 5(41.7%) were negative for Cx43, 4(33.3%) had mild expression for Cx43 and 3(25%) cases had a moderate expression for Cx43. In group IV, 5(71.4%) cases were negative for Cx43, 1(14.3%) had mild staining for Cx43 and 1(14.3%) had intense expression for Cx43. The difference in Cx43 staining intensity between the groups was statistically significant **(p=0.033)**.

Comparison of Cx43 staining in the connective tissue among the study groups (Table VIII& Graph VIII):-

In group I, 1(16.7%) case had no Cx43 expression, 4(66.7%) case had mild staining for Cx43 and 1(16.7%) case had moderate staining for Cx43 in the connective tissue. In group II, 1(7.1%) case had no expression of Cx43,

5(35.7%) cases had mild staining for Cx43, 7(50%) cases had moderate staining for Cx43 and 1(7.1%) case had intense staining for Cx43 in the connective tissue. In group III, 4(33.3%) cases were negative for Cx43 expression, 5(41.7%) cases had mild expression for Cx43 and 3(25%) cases had a moderate expression for Cx43 in the connective tissue. In group IV, 5(71.4%) had mild staining for Cx43, 1(14.3%) case had moderate staining for Cx43 and 1(14.3%) case had intense staining for Cx43. The difference in staining intensity of Cx43 in the connective tissue was not significant (p=0.279).

Comparison of Cx43 staining of suprabasal cell layer among different grades of Oral Submucous Fibrosis (Table IX and Graph IX):-

Among the OSF group, in the very early stage, 1(25%) case had no expression and 3(75%) cases showed mild expression for Cx43. In early stage, 1(14.3%) case showed no expression, 3(42.9%) cases showed mild expression for Cx43 and 3(42.9%) cases showed moderate expression. In the moderately advanced stage, 1(33.3%) case had mild expression for Cx43 and 2(66.7%) cases had moderate expression of Cx43. None of the cases were in the advanced stage of OSF. There was no significant difference in the staining intensity between the different stages of OSF (p=0.431)

Comparison of Cx43 staining of suprabasal cell layer among the different grades of epithelial dysplasia (Table X & Graph X):-

The cases of epithelial dysplasia were graded as mild, moderate and severe. In cases of mild dysplasia, 2(33.3%) cases showed no expression of Cx43, 2(33.3%) cases showed a mild expression of Cx43 and 2(33.3%) cases showed a moderate expression of Cx43. Among the cases of moderate dysplasia, 3(50%) cases showed no expression of Cx43, 2(33.3%) cases showed mild expression of Cx43 and 1(16.7%) case had a moderate staining for Cx43. None of the cases in the study group had severe dysplasia. The difference in staining intensity of the suprabasal layer between different grades of dysplasia was not significant (p=0.766).

Distribution of Cx43 staining of basal and suprabasal cell layers among the study groups. (Table XI & Graph XI):-

Comparing the basal cell layer staining among the study groups, group II showed expression in 4(28.6%) cases**(p=0.047)**. The suprabasal cell layer staining in the study groups showed that , group I had a positive expression in 5(83.3%) cases, group II in 12(85.7%), group III in 7(58.3%) and group IV in 2(28.6%) cases, respectively **(p=0.046)**.

Distribution of Cx43 staining between group II (OSF) and group IV (OSCC with history of OSF) (Table XII & Graph XII):-

In group II, 13(92.9%) cases had expression of Cx43 and 1(7.1%) cases did not express Cx43. In group IV, 6(85.7%) cases had expression of Cx43 and 1 (14.3%) case had no Cx43 expression. The difference in Cx43 staining was not significant between group II and IV (p=1.0).

Comparison of Cx43 staining intensity of basal cell layer between group II (OSF) and group IV (OSCC with history of OSF) (Table XIII & Graph XIII):-

In group II, 10(71.4%) cases showed no expression in the basal cell layer, 3(21.4%) cases showed a mild expression of Cx43 and 1(7.1%) case showed a moderate expression. In group IV were cases of OSF with history of OSCC, all 7(100%) of the cases showed no expression of Cx43 in the basal cell layer. The difference in Cx43 staining of the basal cell layer was not statistically significant (p=0.291).

Comparison of Cx43 staining intensity of suprabasal cell layer between group II (OSF) and group IV (OSCC with history of OSF) (Table XIV & Graph XIV):-

In group II, 2(14.3%) cases showed no expression of Cx43 while 7(50%) cases showed a mild expression and 5(35.7%) cases showed a moderate expression. In group IV, 5(71.4%) cases showed no expression of Cx43, 1(14.3%) case showed

a mild Cx43 expression and 1(14.3%) case had anintense expression of Cx43. The difference in suprabasal layer staining intensity was statistically significant between groups II and IV **(p=0.014).**

Comparison of Cx43 staining intensity of connective tissue between group II (OSF) and group IV (OSCC with history of OSF) (Table XV& Graph XV):-

In group II, there was 1(7.1%) case which showed no expression of Cx43, 5(35.7%) cases showed a mild expression, 7(50%) casesshowed a moderate expression of Cx43 and 1(7.1%) case showed anintense expression of Cx43. In group IV, 5(71.4%) cases showed a mild Cx43 expression while 1(14.3%) case showed moderate expression and 1(14.3%) case showed anintense expression of Cx43. The difference in connective tissue staining between groups II and IV was not significant (p=0.313).

Comparison of Cx43 staining intensity of basal cell layer between group II (OSF) and group III (Epithelial dysplasia) (Table XVI & Graph XVI):-

In group II, 10(71.4%) cases did not show the expression for Cx43, 3(21.4%)showed a mild expression and 1(7.1%) case showed a moderate expression of Cx43. In group III, all 12(100%) cases showed no expression of Cx43. The difference in basal layer staining intensity between the groups II and II was not significant (p=0.132).

Comparison of Cx43 staining intensity of suprabasal cell layer between group II (OSF) and group III (Epithelial dysplasia) (Table XVII & Graph XVII):-

In group II 2(14.3%) cases showed no expression of Cx43, while 7(50%) cases showed a mild expression and 5(35.7%) casesshowed a moderate expression for Cx43. In group III, 5(41.7%) cases showed no expression of Cx43, 4(33.3%) showed a mild expression of Cx43 and 3(25%) showed a moderate expression of Cx43. The difference in suprabasal layer staining between the groups II and III was not significant (p=0.292).

Comparison of Cx43 staining intensity of connective tissue between group II (OSF) and group III (Epithelial dysplasia) (Table XVIII & Graph XVIII):-

In group II, 1(7.1%) showed no expression of Cx43, 5(35.7%) cases showed a mild expression of Cx43, 7(50%) cases showed a moderate expression and 1(7.1%) case showed an intense expression of Cx43. In group III, 4(33.3%) did not show any expression of Cx43, 5(41.7%) cases showed a mild expression of Cx43 and 3(25%) cases showed a moderate expression of Cx43. The difference in connective tissue staining between groups II and III was not significant (p=0.234).

Distribution of Cx43 staining between group III (Epithelial dysplasia) and group IV (OSCC with history of OSF) (Table XIX & Graph XIX):-

In group III, 8(66.7%) cases had an expression of Cx43 while 4(33.3%) had no expression of Cx43. In group IV, 6(85.7%) cases had expression for Cx43 and 1(14.3%) had no expression of Cx43. The difference in Cx43 staining between groups III and IV was not significant (p=0.363).

Comparison of Cx43 staining intensity of basal cell layer between group III (Epithelial dysplasia) and group IV (OSCC with history of OSF) (Table XX & Graph XX):-

In group III and group IV, all 12 (100%) cases and 7 (100%)showed no expression of Cx43(p=1.0).

Comparison of Cx43 staining intensity of suprabasal cell layer between group III (Epithelial dysplasia) and group IV (OSCC with history of OSF)(Table XXI & Graph XXI):-

In group III, 5(41.7%) of cases showed no expression of Cx43, 4(33.3%) of the cases showed a mild expression and 3(25%) of cases showed a moderate expression of Cx43. In group IV, 5(71.4%) cases had no expression of Cx43 while 1(14.3%) cases showed a mild expression of Cx43 and 1(14.3%) showed an intense expression for Cx43. The difference in suprabasal layer staining intensity between groups III and IV was not significant (p=0.186).

Comparison of Cx43 staining intensity of Connective tissue between group III(Epithelial dysplasia) and group IV (OSCC with history of OSF)(Table XXII & Graph XXII):-

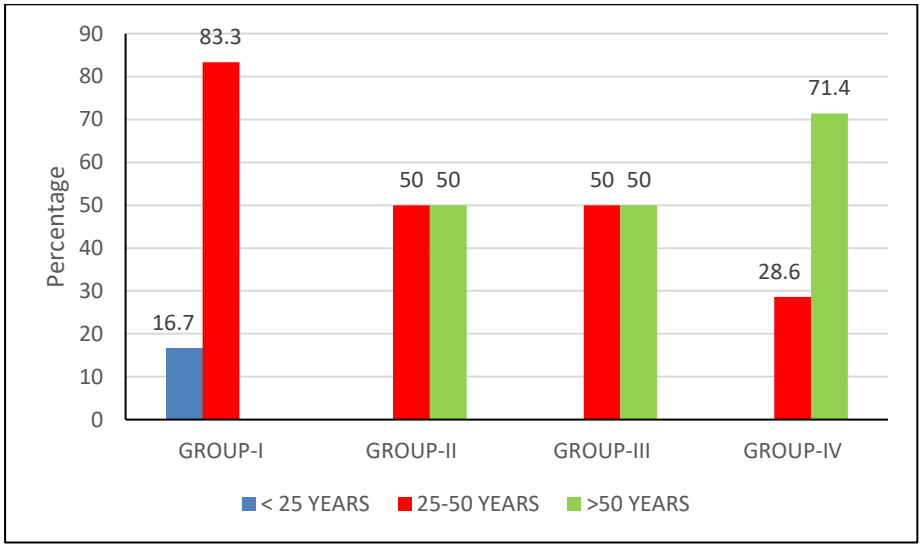
In group III, there were 4(33.3%) cases that showed no expression of Cx43, 5(41.7%) cases showed a mild expression of Cx43 and 3(25%) cases showed a moderate expression for Cx43. In group IV, 5(71.4%) cases showed a mild expression while 1(14.3%) case showed a moderate expression and 1(14.3%) case showed intense expression of Cx43. The difference in connective tissue staining intensity between groups III and IV was not significant (p=0.169).

Tables and Graphs

TABLE I: AGE DISTRIBUTION AMONG THE STUDY GROUPS (N=39)

AGE	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
<25 YEARS	1(16.7%)	0(0%)	0(0%)	0(0%)	0.081
25-30 YEARS	5(83.3%)	7(50%)	6(50%)	2(28.6%)	
>50 YEARS	0(0%)	7(50%)	6(50%)	5(71.4%)	

GRAPH I: AGE DISTRIBUTION AMONG THE STUDY GROUPS (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

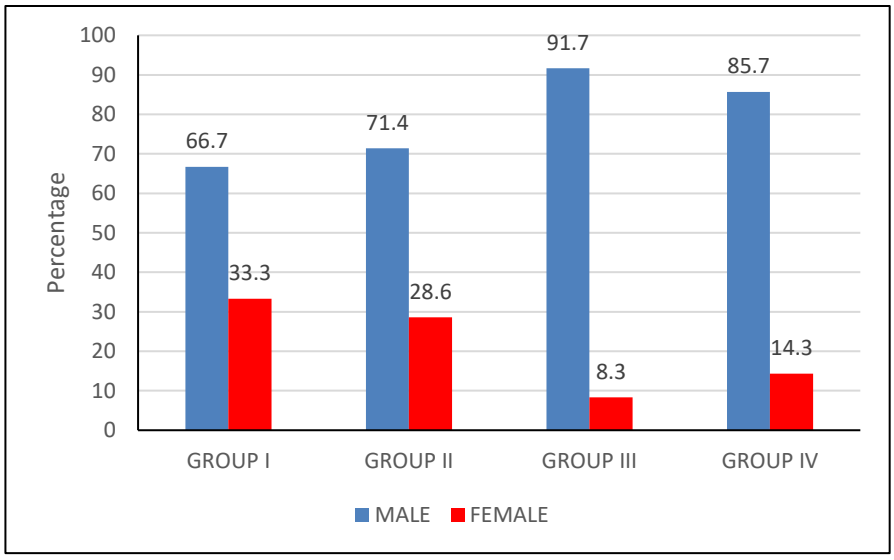
GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE II: GENDER DISTRIBUTION AMONG THE STUDY GROUPS (N=39)

GENDER	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
MALE	4(66.7%)	10(71.4%)	11(91.7%)	6(85.7%)	0.490
FEMALE	2(33.3%)	4(28.6%)	1(8.3%)	1(14.3%)	

1

GRAPH II : GENDER DISTRIBUTION AMONG THE STUDY GROUPS (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE III: DISTRIBUTION OF HABITS AMONG THE STUDY GROUPS (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
NO HABITS	6(100%)	0(0%)	0(0%)	0(0%)	
ARECA NUT CHEWING	0(0%)	9(64.3%)	0(0%)	0(0%)	
TOBACCO CHEWING	0(0%)	0(0%)	7(58.3%)	0(0%)	
ARECA NUT +TOBACCO	0(0%)	5(35.7%)	1(8.3%)	4(57.1%)	
SMOKING+ ARECANUT	0(0%)	0(0%)	4(33.3%)	1(14.3%)	0.000**
SMOKING + TOBACCO CHEWING+ARECA NUT CHEWING	0(0%)	0(0%)	0(0%)	2(28.6%)	

****p<0.01**

GRAPH III: DISTRIBUTION OF HABITS AMONG THE STUDY GROUPS (N=39)

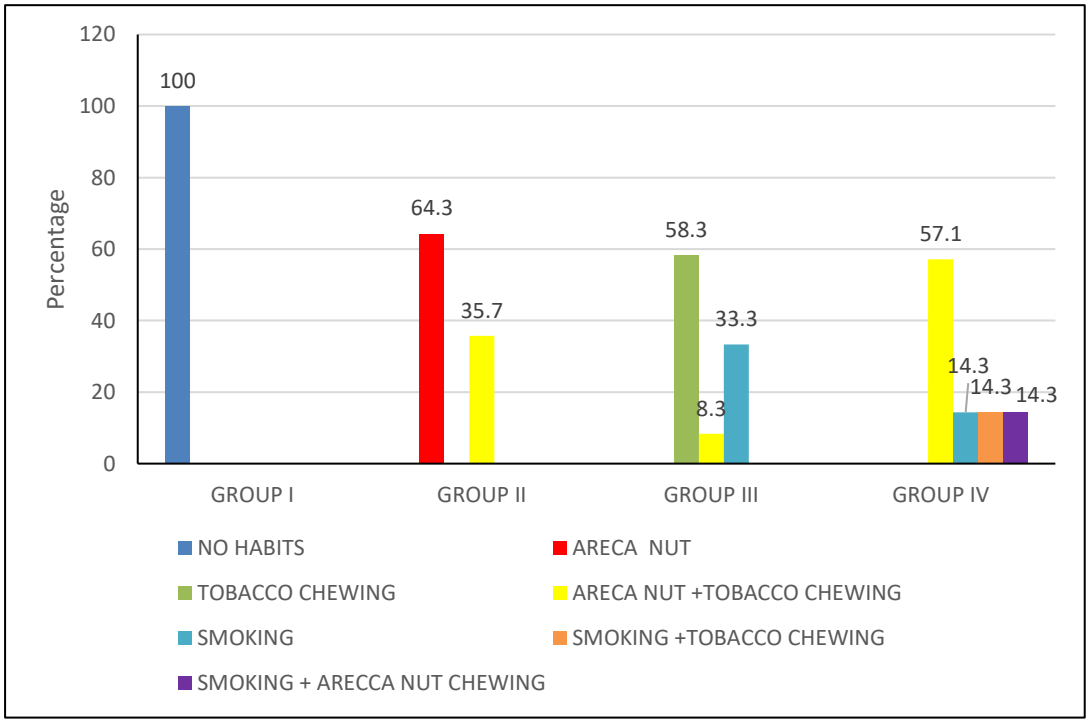
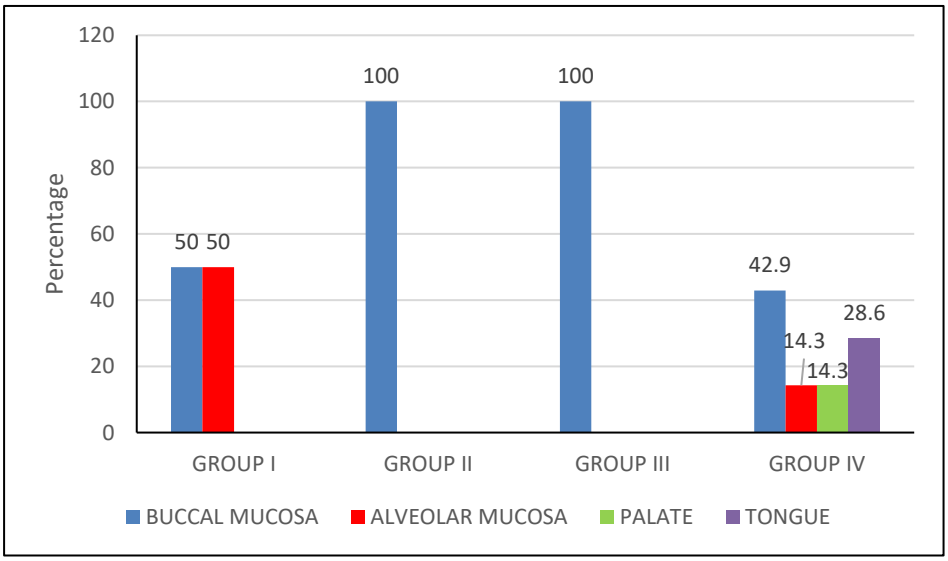


TABLE IV: DISTRIBUTION OF SITE OF LESION AMONG THE STUDY GROUPS (N=39)

SITE	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
BUCCAL MUCOSA	3(50%)	14(100%)	12(100%)	3(42.9%)	0.001**
ALVEOLAR MUCOSA	3(50%)	0(0.0%)	0(0.0%)	1(14.3%)	
PALATE	0(0.0%)	0(0.0%)	0(0.0%)	1(14.3%)	
TONGUE	0(0.0%)	0(0.0%)	0(0.0%)	2(28.6%)	

****p<0.01**

GRAPH IV: DISTRIBUTION OF SITE OF THE LESION AMONG THE STUDY GROUPS (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

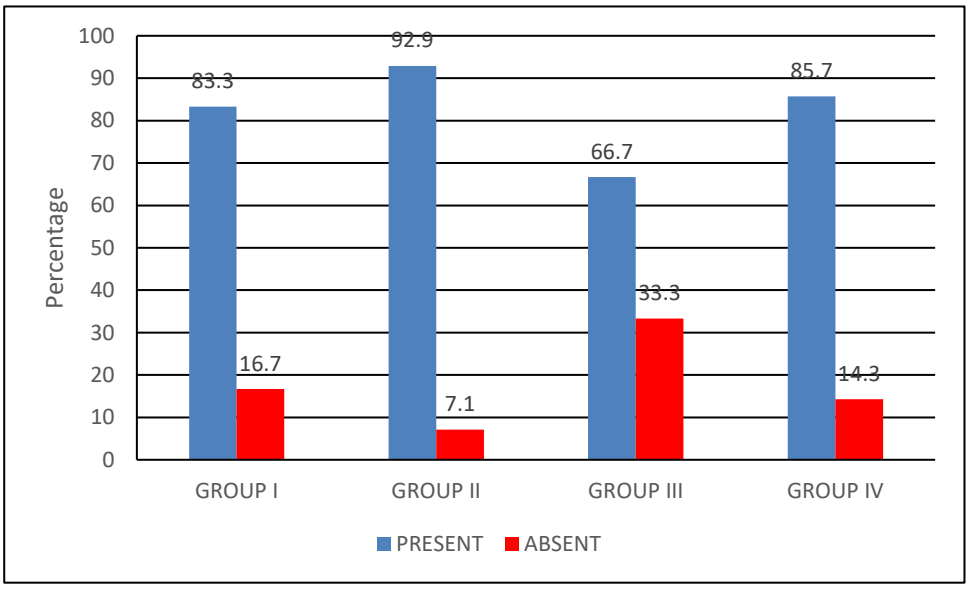
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE V: DISTRIBUTION OF STAINING OF Cx43 AMONG THE STUDY GROUPS (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
PRESENT	5(83.3%)	13(92.9%)	8(66.7%)	6(85.7%)	0.375
ABSENT	1(16.7%)	1(7.1%)	4(33.3%)	1(14.3%)	

GRAPH V: DISTRIBUTION OF STAINING OF Cx43 AMONG THE STUDY GROUPS (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

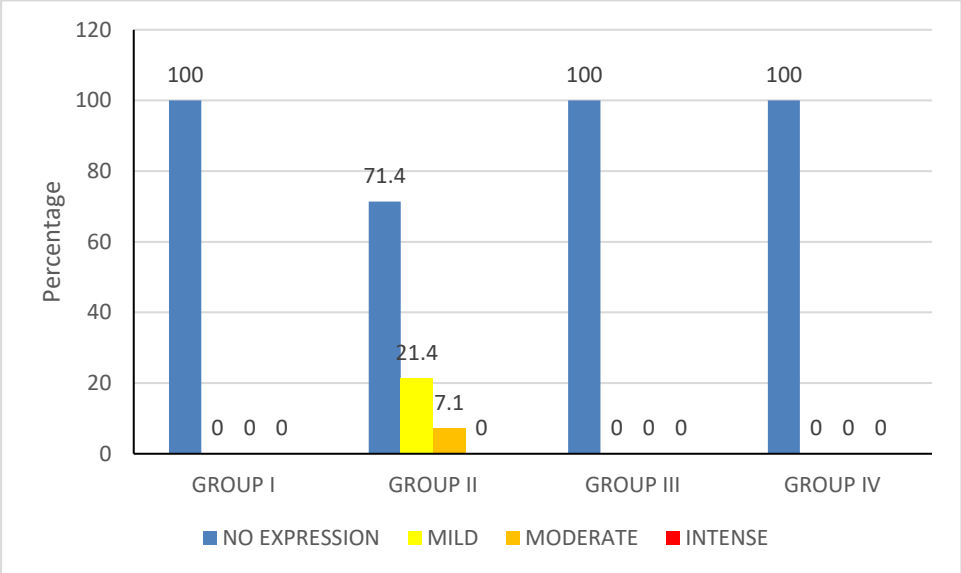
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE VI: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL LAYER AMONG THE STUDY GROUP (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	6(100%)	10(71.4%)	12(100%)	7(100%)	0.241
MILD	0(0%)	3(21.4%)	0(0%)	0(0%)	
MODERATE	0(0.0%)	1(7.1%)	0(0%)	0(0.0%)	
INTENSE	0(0.0%)	0(0%)	0(0.0%)	0(0%)	

GRAPH VI: COMPARISONOF Cx43 STAINING INTENSITY OF BASAL LAYER AMONG THE STUDY GROUP (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

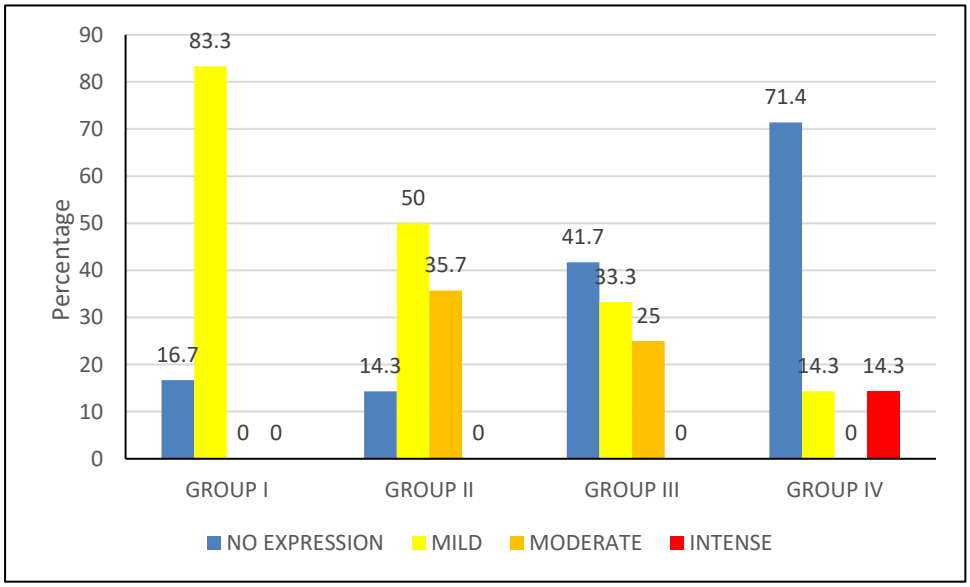
GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE VII: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG THE STUDY GROUP (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	1(16.7%)	2(14.3%)	5(41.7%)	5(71.4%)	0.033*
MILD	5(83.3%)	7(50%)	4(33.3%)	1(14.3%)	
MODERATE	0(0.0%)	5(35.7%)	3(25%)	0(0%)	
INTENSE	0(0.0%)	0(0.0%)	0(0.0%)	1(14.3%)	

*p<0.05

GRAPH VII: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG THE STUDY GROUP (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

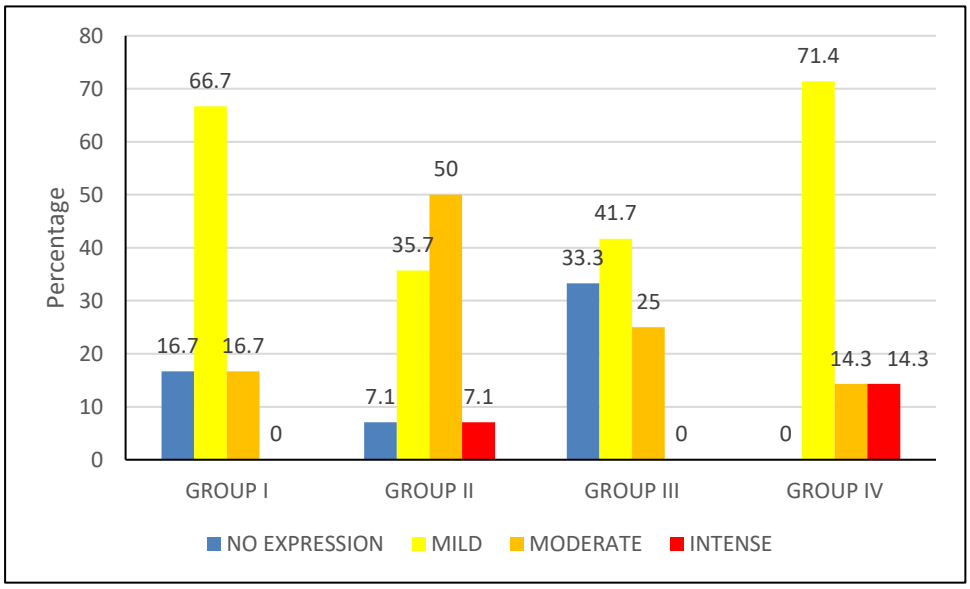
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE VIII: COMPARISON OF Cx43 STAINING INTENSITY IN THE CONNECTIVE TISSUE AMONG THE STUDY GROUP (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	1(16.7%)	1(7.1%)	4(33.3%)	0(0%)	0.279
MILD	4(66.7%)	5(35.7%)	5(41.7%)	5(71.4%)	
MODERATE	1(16.7%)	7(50%)	3(25%)	1(14.3%)	
INTENSE	0(0.0%)	1(7.1%)	0 (0%)	1(14.3%)	

GRAPH VIII: COMPARISON OF Cx43 STAINING INTENSITY IN THE CONNECTIVE TISSUE AMONG THE STUDY GROUP (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE IX: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG DIFFERENT GRADES OF ORAL SUBMUCOUS FIBROSIS (GROUP-II) (N=14)

	VERY EARLY STAGE (n=4)	EARLY STAGE (n=7)	MODERATELY ADVANCED STAGE (n=3)	ADVANCED STAGE (n=0)	p value
NO EXPRESSION	1(25%)	1(14.3%)	0(0%)	0(0%)	0.431
MILD	3(75%)	3(42.9%)	1(33.3%)	0(0%)	
MODERATE	0(0%)	3(42.9%)	2(66.7%)	0(0%)	
INTENSE	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	

GRAPH IX: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG THE DIFFERENT GRADES OF ORAL SUBMUCOUS FIBROSIS (GROUP-II) (N=14)

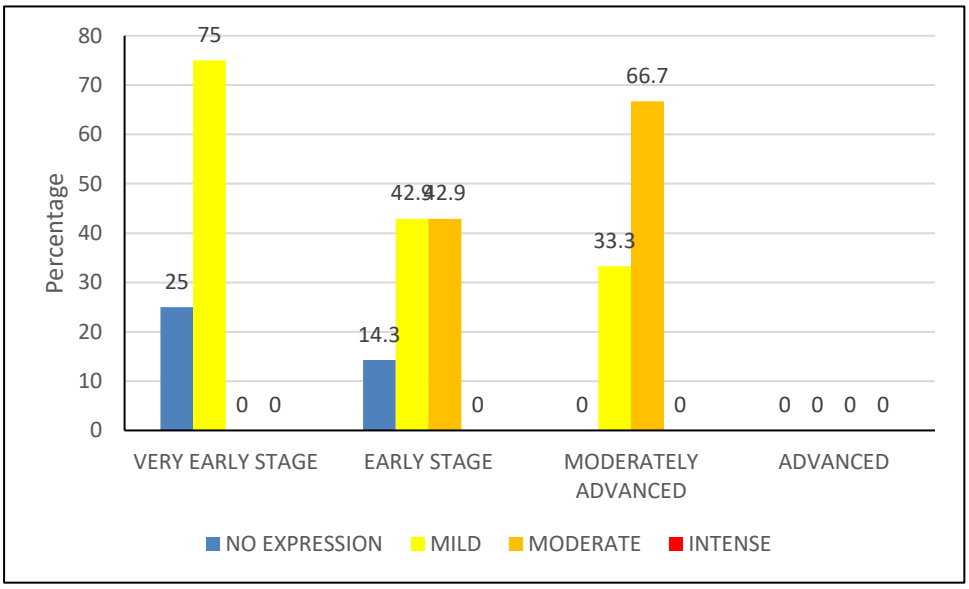


TABLE X: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG DIFFERENT GRADES OF EPITHELIAL DYSPLASIA (GROUP-III) (N=12)

	MILD DYSPLASIA (n=6)	MODERATE DYSPLASIA (n=6)	SEVERE DYSPLASIA (n=0)	p value
NO EXPRESSION	2(33.3%)	3(50.0%)	0(0%)	0.766
MILD	2(33.3%)	2(33.3%)	0(0%)	
MODERATE	2(33.3%)	1(16.7%)	0(0%)	
INTENSE	0(0%)	0(0%)	0(0%)	

GRAPH X: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER AMONG THE DIFFERENT GRADES OF EPITHELIAL DYSPLASIA (GROUP-III) (N=12)

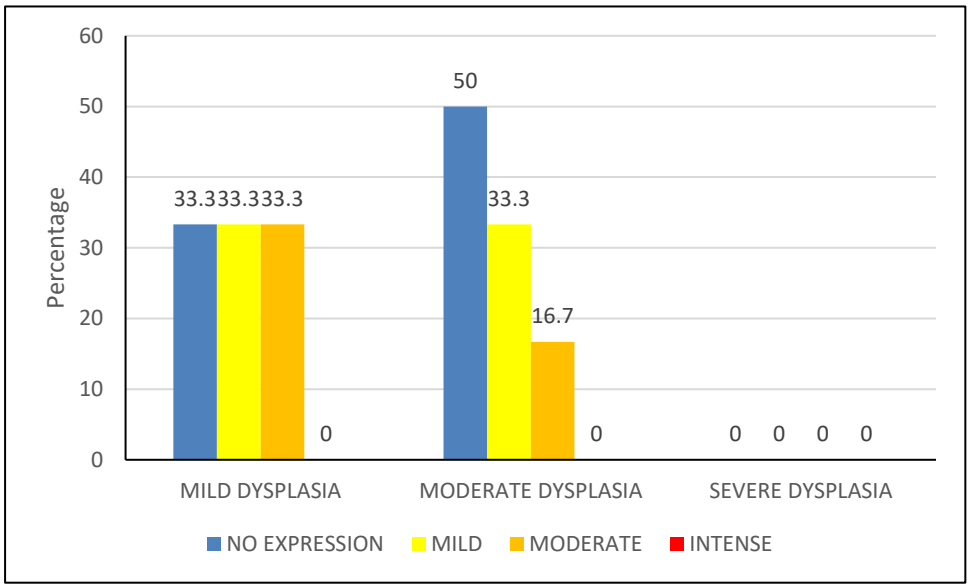
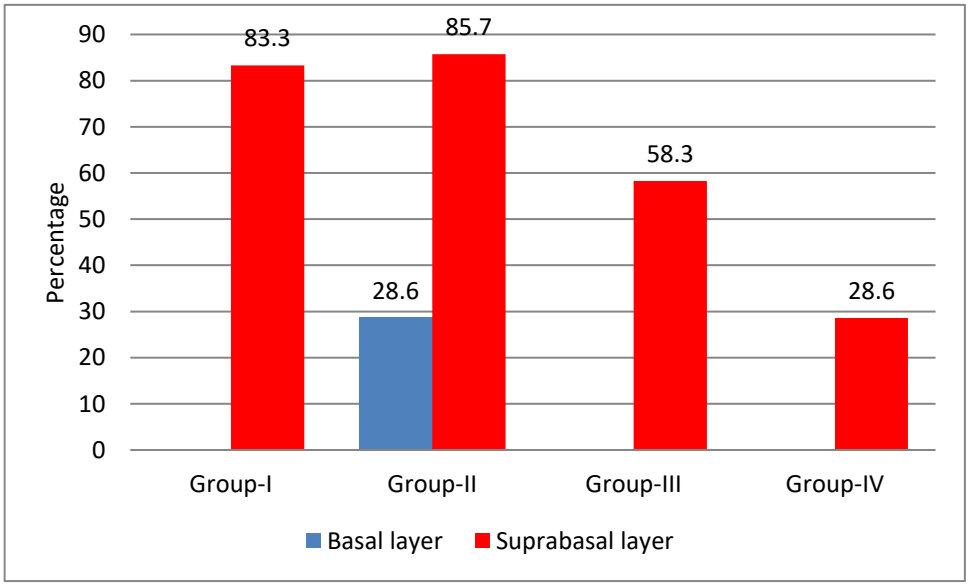


TABLE XI: DISTRIBUTION OF Cx43 STAINING OF BASAL AND SUPRABASAL CELL LAYERS AMONG THE STUDY GROUPS (N=39)

	GROUP I (n=6)	GROUP II (n=14)	GROUP III (n=12)	GROUP IV (n=7)	p value
BASAL CELL LAYER	0(0%)	4(28.6%)	0(0%)	0(0%)	0.047*
SUPRABASAL CELL LAYER	5(83.3%)	12(85.7%)	7(58.3%)	2(28.6%)	0.046*
BOTH THE CELL LAYERS	0(0%)	4(100%)	0(0%)	0(0%)	
ABSENT IN BOTH THE CELL LAYERS	1(7.7%)	2(15.4%)	5(38.5%)	5(38.5%)	

GRAPH XI : DISTRIBUTION OF Cx43 STAINING OF BASAL AND SUPRABASAL CELL LAYERS AMONG THE STUDY GROUPS (N=39)



GROUP I : NORMAL MUCOSA (n=6)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

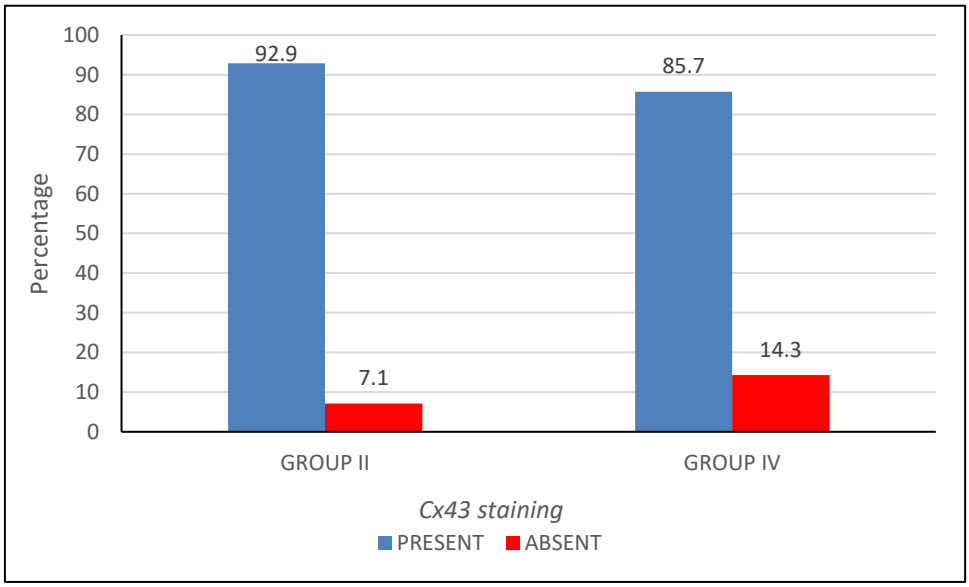
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSCC WITH HISTORY OF OSF (n=7)

TABLE XII: DISTRIBUTION OF STAINING OF Cx43 BETWEEN GROUP II AND GROUP IV (N=21)

	GROUP II (n=14)	GROUP IV (n=7)	p value
PRESENT	13(92.9%)	6(85.7%)	1.0
ABSENT	1(7.1%)	1(14.3%)	

GRAPH XII: DISTRIBUTION OF STAINING OF Cx43 BETWEEN GROUP II AND GROUP IV (N=21)



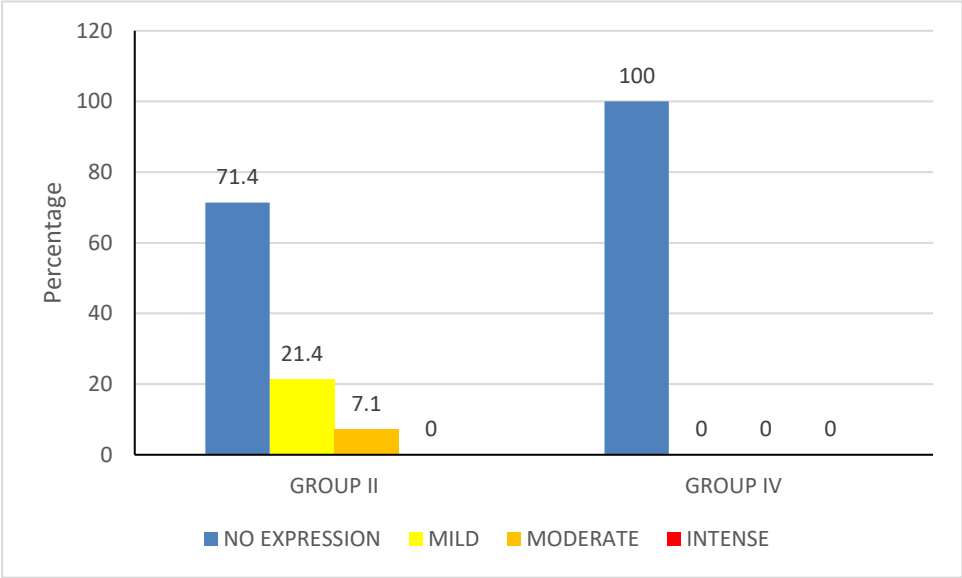
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP IV: OSCC WITH HISTORY OF OSF (n=7)

TABLE XIII: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP II AND GROUP IV (N=21)

	GROUP II (n=14)	GROUP IV (n=7)	p value
NO EXPRESSION	10(71.4%)	7(100%)	0.291
MILD	3(21.4%)	0(0%)	
MODERATE	1(7.1%)	0(0.0%)	
INTENSE	0(0%)	0(0%)	

GRAPH XIII: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP II AND GROUP IV (N=21)



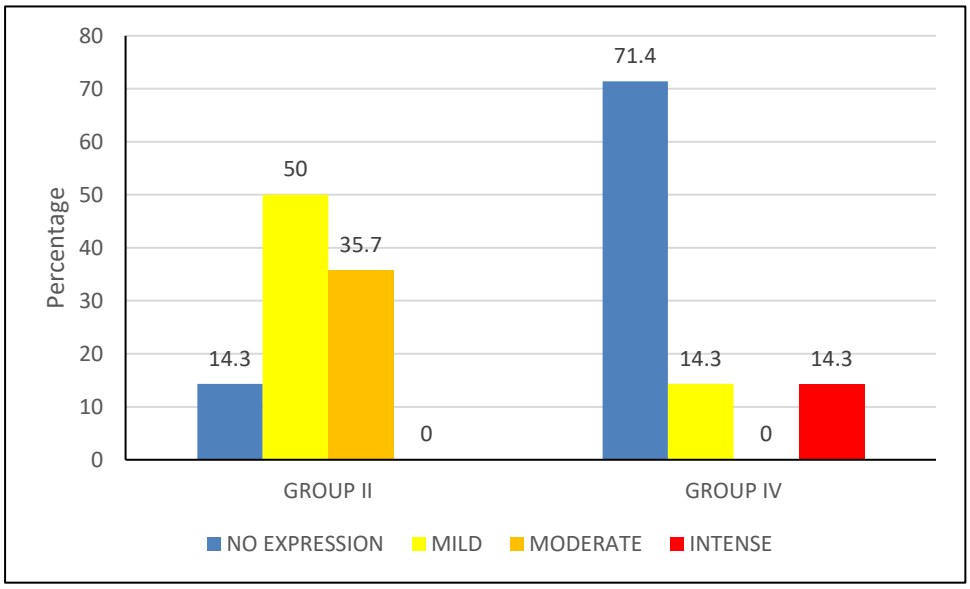
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP IV: OSCC WITH HISTORY OF OSF (n=7)

TABLE XIV: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER BETWEEN GROUP II AND GROUP IV (N=21)

	GROUP II (n=14)	GROUP IV (n=7)	p value
NO EXPRESSION	2(14.3%)	5(71.4%)	0.014*
MILD	7(50%)	1(14.3%)	
MODERATE	5(35.7%)	0(0%)	
INTENSE	0(0.0%)	1(14.3%)	

GRAPH XIV: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER BETWEEN GROUP II AND GROUP IV



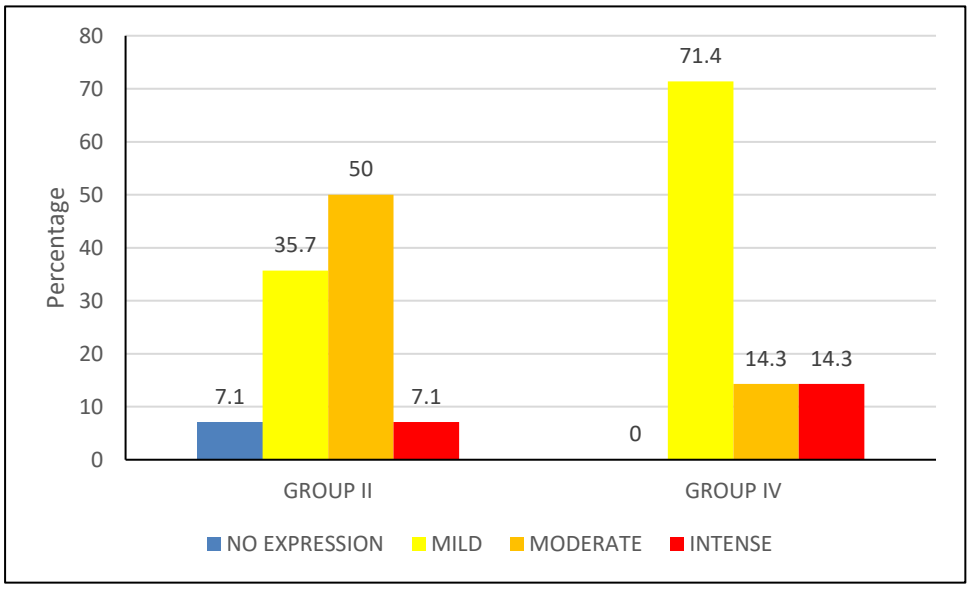
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP IV: OSCC WITH HISTORY OF OSF (n=

TABLE XV: COMPARISON OF Cx43 STAINING INTENSITY IN THE CONNECTIVE TISSUE BETWEEN GROUP II AND GROUP IV (N=21)

	GROUP II (n=14)	GROUP IV (n=7)	p value
NO EXPRESSION	1(7.1%)	0(0%)	0.313
MILD	5(35.7%)	5(71.4%)	
MODERATE	7(50%)	1(14.3%)	
INTENSE	1(7.1%)	1(14.3%)	

GRAPH XV: COMPARISON OF Cx43 STAINING INTENSITY IN THE CONNECTIVE TISSUE BETWEEN GROUP II AND GROUP IV (N=21)



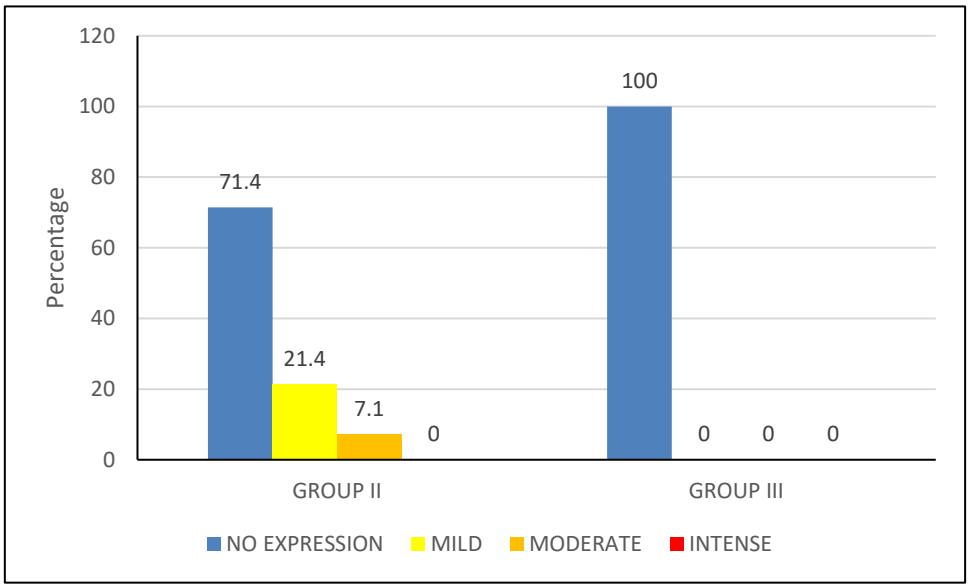
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP IV: OSCC WITH HISTORY OF OSF (n=7)

TABLE XVI: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP II AND GROUP III (N=21)

	GROUP II (n=14)	GROUP III (n=12)	p value
NO EXPRESSION	10(71.4%)	12(100%)	0.132
MILD	3(21.4%)	0(0%)	
MODERATE	1(7.1%)	0(0%)	
INTENSE	0(0.0%)	0(0%)	

GRAPH XVI: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP II AND GROUP III



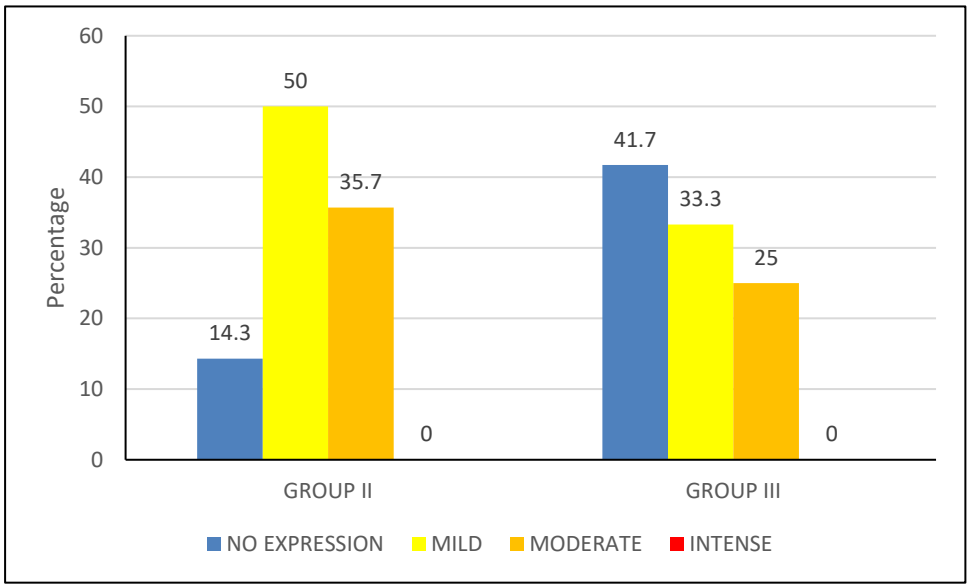
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

TABLE XVII: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER BETWEEN GROUP II AND GROUP III (N=26)

	GROUP II (n=14)	GROUP III (n=12)	p value
NO EXPRESSION	2(14.3%)	5(41.7%)	0.292
MILD	7(50%)	4(33.3%)	
MODERATE	5(35.7%)	3(25%)	
INTENSE	0(0.0%)	0(0%)	

GRAPH XVII: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER BETWEEN GROUP II AND GROUP III



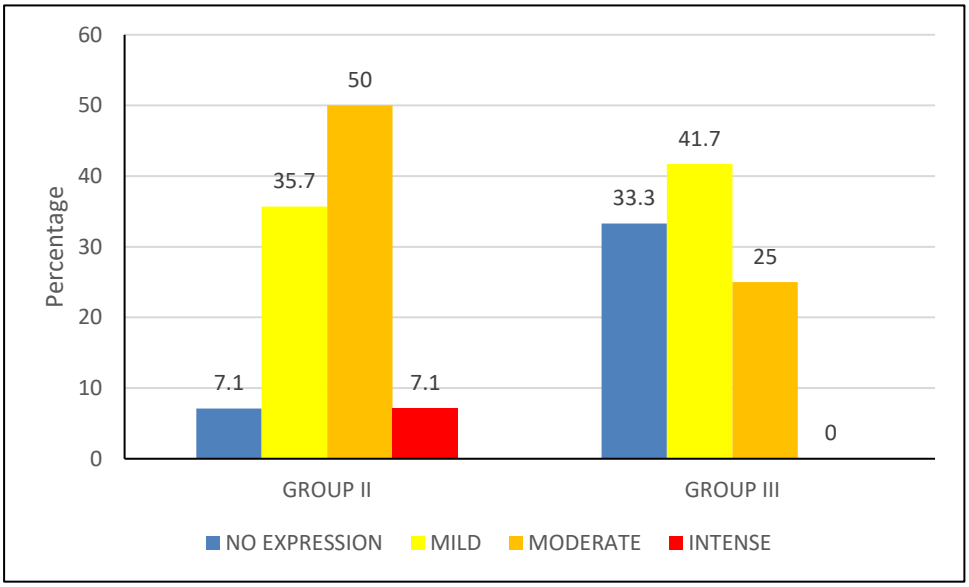
(N=26)

GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III : EPITHELIAL DYSPLASIA (n=12)

TABLE XVIII: COMPARISON OF Cx43 STAINING INTENSITY OF CONNECTIVE TISSUE INTENSITY BETWEEN GROUP II AND GROUP III (N=26)

	GROUP II (n=14)	GROUP III (n=12)	p value
NO EXPRESSION	1(7.1%)	4(33.3%)	0.234
MILD	5(35.7%)	5(41.7%)	
MODERATE	7(50%)	3(25%)	
INTENSE	1(7.1%)	0(0%)	



GRAPH XVIII: COMPARISON OF Cx43 STAINING INTENSITY OF CONNECTIVE TISSUE BETWEEN GROUP II AND GROUP III (N=26)

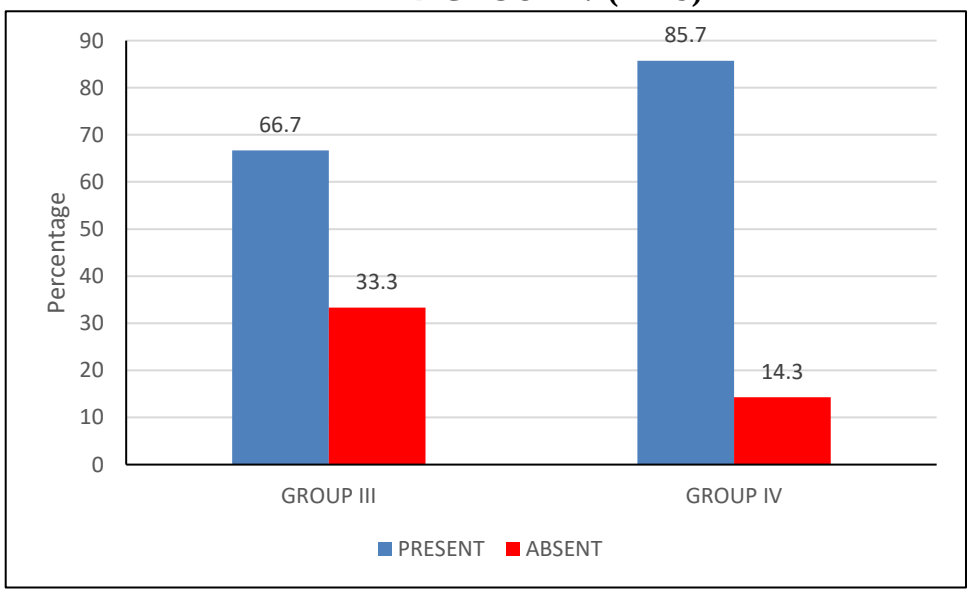
GROUP II : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP III: EPITHELIAL DYSPLASIA (n=12)

TABLE XIX: DISTRIBUTION OF Cx43 STAINING BETWEEN GROUP III AND GROUP IV (N=19)

	GROUP III (n=14)	GROUP IV (n=12)	p value
PRESENT	8(66.7%)	6(85.7%)	0.363
ABSENT	4(33.3%)	1(14.3%)	

GRAPH XIX: DISTRIBUTION OF Cx43 STAINING BETWEEN GROUP III AND GROUP IV (N=19)



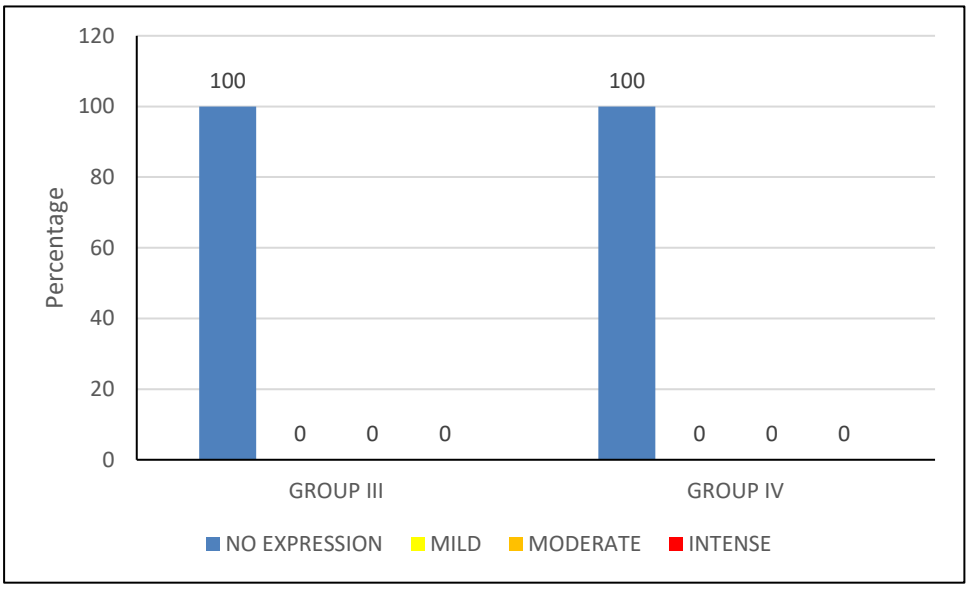
GROUP III : ORAL SUBMUCOUS FIBROSIS (n=14)

GROUP IV : OSF WITH HISTORY OF OSCC (n=7)

TABLE XX: COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP III AND GROUP IV (N=19)

	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	12(100%)	7(100%)	1.0
MILD	0(0%)	0(0%)	
MODERATE	0(0%)	0(0%)	
INTENSE	0(0.0%)	0(0%)	

GRAPH XX - COMPARISON OF Cx43 STAINING INTENSITY OF BASAL CELL LAYER BETWEEN GROUP III AND GROUP IV



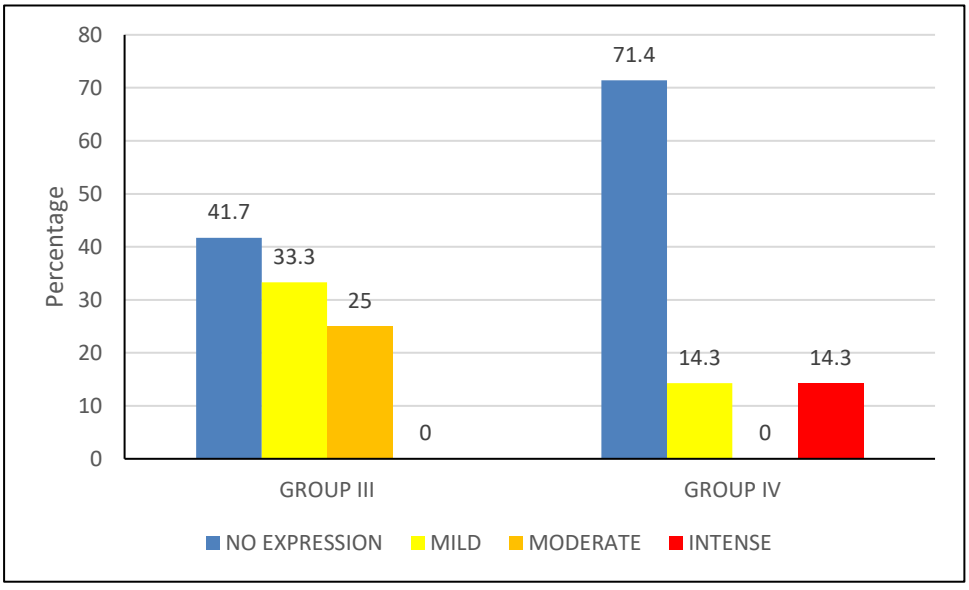
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSF WITH HISTORY OF OSCC (n=7)

TABLE XXI: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER INTENSITY BETWEEN GROUP III AND GROUP IV (N=19)

	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	5(41.7%)	5(71.4%)	0.186
MILD	4(33.3%)	1(14.3%)	
MODERATE	3(25%)	0(0%)	
INTENSE	0(0.0%)	1(14.3%)	

GRAPH XXI: COMPARISON OF Cx43 STAINING INTENSITY OF SUPRABASAL CELL LAYER BETWEEN GROUP III AND GROUP IV (N=19)



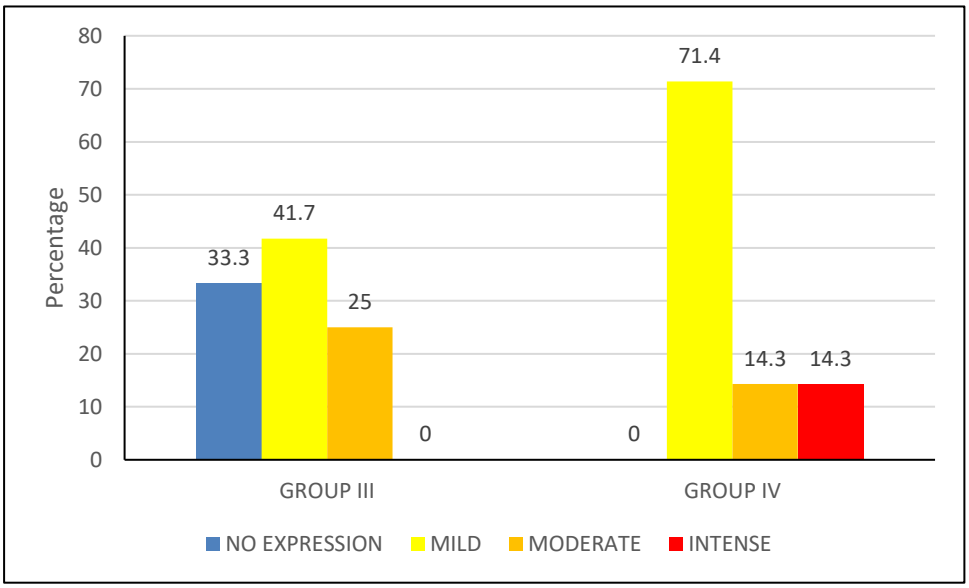
GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSF WITH HISTORY OF OSCC (n=7)

TABLE XXII: COMPARISON OF Cx43 STAINING INTENSITY OF CONNECTIVE TISSUE BETWEEN GROUP III AND GROUP IV (N=19)

	GROUP III (n=12)	GROUP IV (n=7)	p value
NO EXPRESSION	4(33.3%)	0(0%)	0.169
MILD	5(41.7%)	5(71.4%)	
MODERATE	3(25%)	1(14.3%)	
INTENSE	0(0.0%)	1(14.3%)	

GRAPH XXII: COMPARISON OF Cx43 STAINING INTENSITY OF CONNECTIVE TISSUE BETWEEN GROUP III AND GROUP IV (N=19)



GROUP III : EPITHELIAL DYSPLASIA (n=12)

GROUP IV : OSF WITH HISTORY OF OSCC (n=7)

Photographs

COLOUR PLATE I

ARMAMENTARIUM

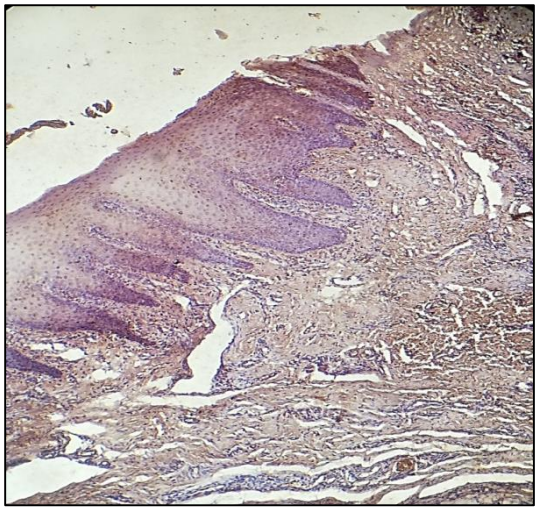
ANTIBODY KIT

PRIMARY ANTIBODY

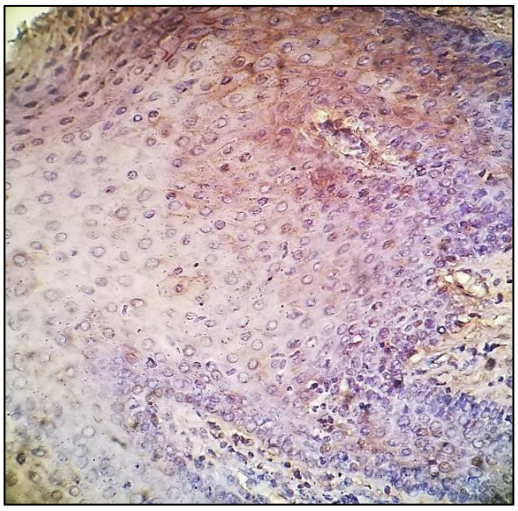
SECONDARY ANTIBODY

COLOUR PLATE II

Cx43 expression in normal mucosa

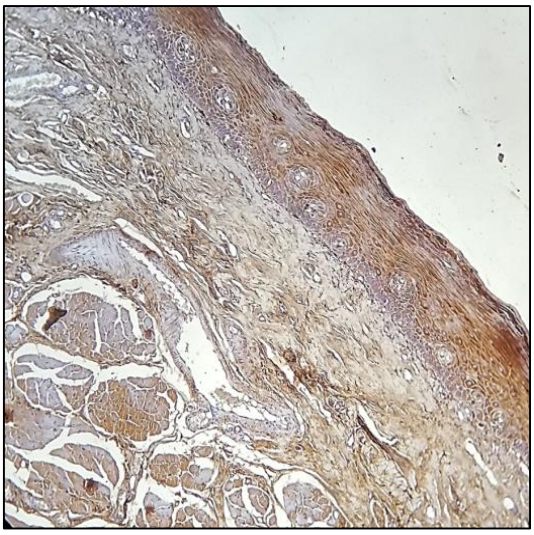


10x

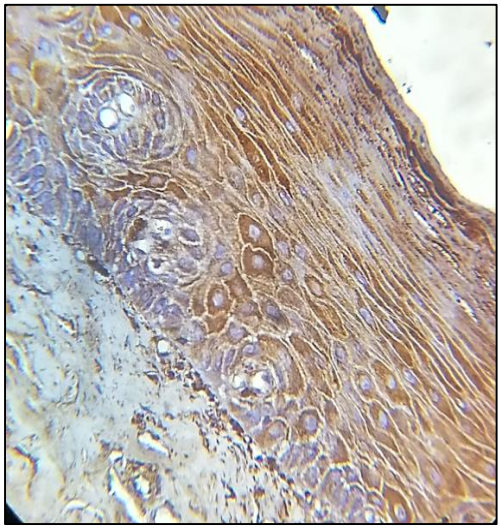


40x

Cx43 expression in OSF



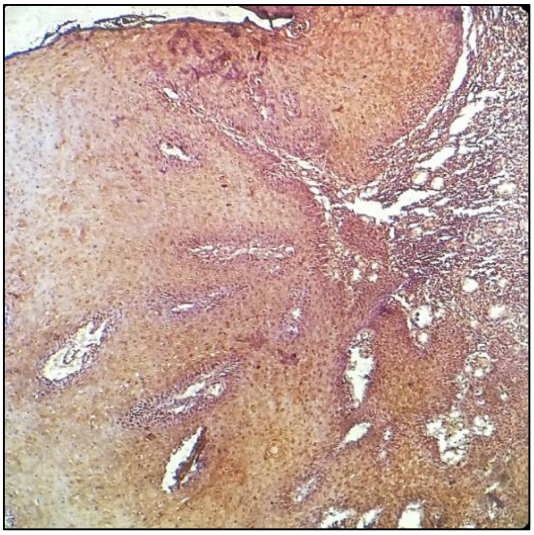
10x



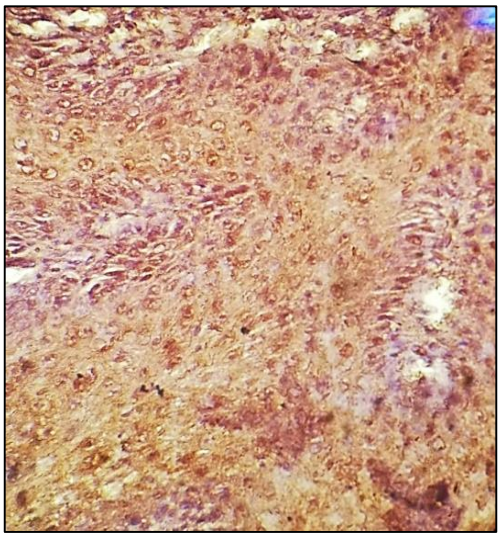
40x

COLOUR PLATE III

Cx43 expression in dysplasia

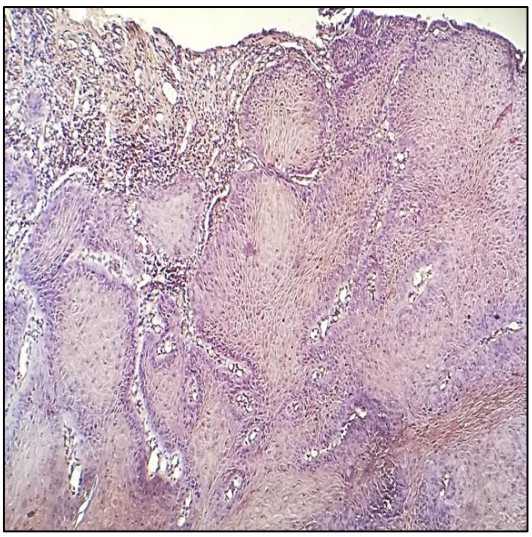


10x

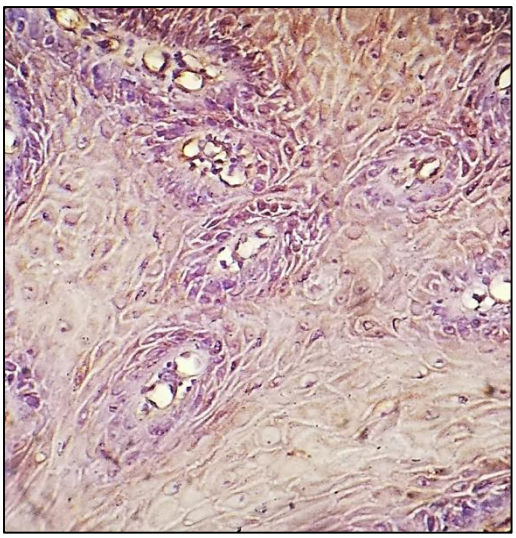


40x

Cx43 expression in OSCC with history of OSF



10x



40x

Discussion

Oral squamous cell carcinoma (OSCC) accounts for almost 90% of the malignancies that occur in the head and neck regions.[74]They are seen in various parts of the oral cavity such as buccalmucosa, tongue, floor of the mouth, palate and the oropharyngealregions.[75]OSCC that are seen in the South-East Asian countries are frequently associated withthe habits of areca nut chewing, tobacco chewing and alcohol consumption. The incidence of OSCC is on the rise and they have a reduced over-all 5 year survival rate as majority of the cancers are diagnosed at an advanced stage.[76]

OSCCs are precededby lesions, now referred as Oral Potentially Malignant Disorder (OPMDs) that carry a risk for malignant transformation.[33]TheseincludeLeukoplakia, Erythroplakia and Oral submucous fibrosis (OSF). Not all of these lesions proceed to oral squamous cell carcinoma.Among them,Leukoplakia which refers to white patches seen clinically are one of the most commonly seen OPMD. It has a global prevalence rate of 2.6%, with a malignant transformation rate of 1.36% per year.[77]Histopathologically, the condition exhibits dysplastic features in the epithelium. It is frequently associated with the habit of tobacco chewing and smoking. Buccal mucosa is the most commonly involved site for epithelial dysplasia.[78] The epithelial dysplasia observed histologically can be graded into mild, moderate and severe based on the cytological and architectural features based on the WHO criteria.[3]

OSF is very common in the Indian sub-continent and is associated with the habit of areca nut chewing.[19]Various studies have been carried out to understand the mechanism of the underlying fibrosis. OSF is characterized by stiffening and formation of palpable fibrotic bands that leadto a reduction in mouth opening.

Histopathologically, OSF is a connective tissue disorder characterized by excessive collagen deposition and juxta-epithelial hyalinization. The epithelium associated with OSFwas thought to be atrophic but it has been suggested that as OSF advances, the features of epithelial dysplasia are more evident in the epithelium.[10] Different stages of OSF demonstrate varying epithelial thickness with features of basal cell hyperplasia and hyperkeratosis.[79]

Connexins are trans-membranous proteins that connect the cytoplasm of one cell to the other and form the gap junction. These junctions are responsible for the communication between adjacent cells in sharing secondary messengers, growth factors and ions. In humans, 21 isoforms of connexins have been identified. Among them, Connexin43 (Cx43) has been found to be expressed in majority of the tissues.[52]Together with each connexon molecule, they form GJIC (Gap Junctional Intercellular Communication Channel), through which secondary messengers, ions and growth factors are exchanged between the adjacent cells. Reduction of gap junctions and their channels play an important role in tumorinitiation, progression and migration of the neoplastic cells, thereby facilitating cellular invasion and metastasis.[73]In this study, weanalysed the pattern of Cx43expression among OSF cases, comparing them with that of the expression seen in epithelial dysplasia, in cases of OSCC that were associated with a historyof OSF and normal mucosa which is the control group.

Archival blocks were used as samples in the study groups. There was a significant difference in the distribution of age among the study groups. We observed thatin group I (control group), 1(17%) cases was< 25 years of age and 5(83.3%) cases were between 25-50 years of age. In group II, (OSF), there were 7(50%) cases within 25-50 years of age and 7(50%) cases were > 50 years of age.

The occurrence of OSF in the younger age group findings was consistent with the findings of the study by ***Ranganathan et al*** who stated that OSF was predominantly seen in a younger population.[19] In group III (epithelial dysplasia), 6(50%) cases were within 25-50 years of age and 6(50%) cases were > 50 years of age.This was in agreement by findings of the study by ***Silverman et al*** who described that epithelial dysplasia iscommon in the younger age group, among those associated with the habit of tobacco chewing and smoking which continues to be persistent for many years to continuation of the tobacco chewing habit.[78] In group IV (OSCC with history of OSF), 5(71.4%) cases were> 50 years of age.This finding was consistent with the findings of the study done by ***Mehrotraet al***who stated that OSCC prevalence was likely to be more, in those above 50 years of age due to the long duration of chewing habit.[80] There were 2(28.6%) cases in the age 25-50 years of age. This finding was in agreement with the findings of studies by ***Chaturvedi et al*** and ***Sarode et al*** who stated that OSCC arising in the background of OSF was common in the younger age group.[36,81] Recently, ***Balaji et al***had reported one case of well-differentiated OSCC, in a 32-year old male with a history of OSF and areca-nut chewing.[82]

Considering the gender distribution,male population were predominantly seen in all study groups. In group II (OSF),a majority of the cases (71.4%) were males. This was in line with the findings of the study done by ***Hazarey et al*** who reported a similar male to female ratio of 4.9:1 and ***Ranganathan et al*** who had also observed a male to female ratio of 6:1 in a clinico-pathological study of OSF cases.[83,79] In group III (epithelial dysplasia),91.7% of cases were males. This was in agreement with the findings of the study by ***Petti et al*** who observed a male predilection for epithelial dysplasia, owing to the higher prevalence of tobacco

chewing and cigarette smoking habits among them.[77]In group IV, there were 6(85.7%) males diagnosed with OSCC with a history of OSF. This was in agreement with the findings of the study done by ***Sarode et al*** who stated that given the prevalence of areca nut chewing habit more among males, compared to females, the occurrence of OSF with OSCC showed the same trend.[81]

OSF is commonly seen in those with the habit of areca nut chewing as the constituents of areca nut is thought to promote the fibrotic pathogenesis.Thedistribution of type of habits between the study groups was found to be statistically significant.in group II (OSF)9(64.3%) cases had the habit of areca nut chewing. This finding was consistent with studies by ***Sinor PN et al*** and ***Ranganathan et al*** who had stated that OSF was predominantly associated with the habit of areca nut chewing.[84,79] In group III (epithelial dysplasia), 7(58.3%) cases had the habit of tobacco chewing while 4(33.3%) caseshad the habit of smoking along with arecanut chewing. This was in agreement with the findings of the study by ***Silverman et al***who observed that majority of the population who had epithelial dysplasia were associated with the habit of tobacco usage**.**[78]In group IV (OSCC with history of OSF),4(57%) cases had the habit of chewing areca nut with tobacco, 1(14.3%) case had the habit of smoking, concurrent with areca-nut and tobacco chewing and 1(14.3%) case had the habit of areca nut chewing and smoking. This finding was in agreement with ***IARC*** statement that the mixture of tobacco products with areca nut could increase the risk of malignancy.[5]

In this study, the most common site of the lesions was the buccal mucosa. In group II (OSF) and in group III (epithelial dysplasia) all the cases were from buccal mucosa and 12(100%) of the cases of group III (epithelial dysplasia) were

from buccal mucosa. This finding was in agreement with the study by ***Kanodia et al***who stated that majority of the cases of OSF occurred in the buccal mucosa due to long term contact of areca nut with buccal mucosa and ***Petti et al*** who described that epithelial dysplasia were more prevalent in the buccal mucosa.[77,85]In group IV (OSCC with history of OSF), 3(42.9%) cases were from buccal mucosa, 1(14.3%) from alveolar mucosa, 1(14.3%)was from the palate and 2(28.6%) cases from the tongue.The pattern of distribution of the site of the lesion between the groups was statistically significant which could be related for the prevalence of the particular habit, which could be considered as the etiological event (p=0.001).

Oral epithelial cells displayed intercellular gap junctions just as most other epithelial cells.In their study, ***Xia et al*** stated that in normal epithelia, Cx43 staining was mainly seen in the cell membrane and was weak in the basal cell layer. The staining was also seen more predominantly in stratum spinosum and stratum granulosum and was negative in the stratum corneum.[68]So far, there are no published reports, analyzing the Cx43 expression in oral submucous fibrosis and oral epithelial dysplasia.

On examining the overall staining pattern of Cx43 among the study groups, in Group 1 (Control group), 83% of cases expressed Cx43. In Groups I that is the control group, the absence of the staining can be attributed to the fact that the tissue was obtained from inflamed gingiva. This was in agreement with findings of ***Fernandez-Cobo et al*** who observed that gap junctional proteins were lost in inflammatory process. In group II, 93% of the cases expressed Cx43 staining, the once case which did not express in the epithelium was found to have moderate dysplasia.[86]

In Group III (epithelial dysplasia), expression for Cx43 was seen in 67% of the cases and in 4 cases (33 %) there was no expression and when we analysed the histopathology of these four cases, we found that they presented with moderate dysplasia of the epithelium. ***Trond Aasen et al*** reported the loss of gap junctional protein Cx43in dysplastic cervix and stated that this was primarily due to the reduced expression.[87] We extend the above explanation for the absence of expression in once case of group II, since the epithelium of the OSF case was dysplastic. In Group IV, only one case showed Cx43 expression in both the surface epithelium and in the infiltrating islands in the connective tissue.

In group II, 5 cases expressed Cx43 in the connective tissue and was in close proximity to the epithelial cells. The expression in the connective tissue is explained as follows, in addition to the gap junction function, connexins have also been found to have transcriptional function which is independent of the intercellular communication process.[88] The authors reported that cell mediated contact signalling is important in carcinogenesis. They also stated that epithelial stromal interactions and signalling are also critical for normal cell biology, cancer cell migration and for the expression of connexins in the connective tissue in neoplastic progression.

When we analysed the expression of Cx43 in different layers of the epithelium, we found that none of the cases expressed positivity in the basal layer in Group I, Group III and Group IV while in Group II, 4 cases showed mild to moderate expression (29%). The absence of expression in the basal layer of the control tissue can be explained given the fact the basal cells of the normal epithelia might have developed less gap junctions than the cells in the stratum spinosum and granulosum.[68]The absence of staining in Group III and IV could be

explainedby the hypothesis of ***Xia et al*** that dysplastic epithelium displayed decreased Cx43 expression which parallel the progression of oral carcinogenesis.[68]Given the above fact, the presence of staining in 29% of Group II cases may reflect the alteration in the basal layer staining compared to the normal epithelia. In these 4(29%) of the cases of OSF group, the epithelium was moderately dysplastic. This finding could be a key factor in the progression of dysplasia to malignancy. Supra-basal staining of Cx43 was observed in all the groups and the difference in the percentage of positivity of cases was statistically significant between the groups.83% of controls (Group I) expressed Cx43 in the supra basal layer, but were of only mild intensity, as stated by ***Xia et al***.

The percentage of positivity was maximum in OSF group (group II)(86%), of which 36% had moderate intensity. In our study, in group II, it was interesting to find that, four cases (28%)expressed staining in both basal and supra basal layers and this pattern of staining throughout the thickness of the epithelium was unique to Group II (OSF).We compared the Cx43 expression among the OSF cases which were graded according to the histopathological criteria given by ***Pindborg & Sirsat et al*** as very early stage, early stage, moderately early stage and advanced stage (Table IX).[13]When we analyzed the staining intensity of Cx43 among the different grades of OSF, the distribution of intensity of staining was not statistically significant.

In group III, 58% of the cases expressed staining, of which 25% had moderate intensityand there was no statistically significant difference between grades of dysplasia (Table X).The loss of Cx43 expression seen in dysplastic conditions was studied by ***Mese et al*** who stated that during early stages of the

dysplastic conditions, the gap junctions try to establish homeostasis by exchange of messengers and growth factors between normal adjacent cells.[89] In our study, we did not find a statistically significant difference in the Cx43 expression in the supra-basal layer between the different stages of OSF and different grades of dysplasia.

With regard to the expression of Cx43 in Group IV (OSCC), only two cases (28.6%) showed Cx43 expression in both the surface epithelium and the infiltrating epithelial islands in the connective tissue. The remaining 6 cases (71.4%) exclusively showed Cx43 positivity in the fibroblasts of the connective tissue only.

Among the groups, the connective tissue expression of Cx43 was mild in groups I (normal mucosa), III (epithelial dysplasia) and IV (OSCC with OSF) while in group II, moderate intensity of staining was seen in 50% of cases which was more than that observed in group III (25%), group IV(14.3%) and group I (16.7%). This high expression of Cx43 in the connective tissue was observed by ***Pistorio et al*** who stated that GJIC might allow exchange of αSMA between the fibroblast and mast cells, resulting in increased fibrosis.[90] This increased acquisition of αSMA, a mesenchymal marker, transferred through the GJIC between the fibroblasts might predispose the basal cell layer to a future type II epithelial-mesenchymal transitional event.[91]

Inour study, the expression of Cx43 was observedin two cases of OSCC. In studies done by ***Tanaka et al, Tang et al***, and ***Juan Xia et al.,*** there was a decrease in Cx43 expression,associated with carcinogenesis.[62,64,68] 1(50%) case of a moderately differentiated OSCC showed an intense expression of Cx43 in the

connective tissue. The presence of intense staining in this case could be explained by the findings of ***Han et al*** who stated that increased expression levels of Cx43 is seen in the advancing stages of colonic carcinoma which supports the role of Cx43 in tumor progression and metastatic capacity.[88]***Jamieson et al*** in their study also had found increased expression ofCx43levels in breast cancer.[92]

When we compared the basal layer staining across the groups, 4 cases of group II (29%)showed expression of Cx43 and there was no staining observed in groups I, III and IV (Table XI, p=0.047). These four cases in group II, were also found to show the expression in the supra-basal layer. In all the other groups, only the supra-basal layer expressed Cx43 (Group I: 83%,group III: 58% and Group IV: 29%). This difference in staining pattern was statistically significant. (p = 0.046).We also observed that only in group II (OSF), the staining pattern was seen in the basal and the supra basal layers corresponding to the entire thickness of the epithelium.

When we compared the distribution of Cx43 expression and staining intensity of the basal cell layer, between groups II and IV, there was no significant difference; but when we compared the expression in the supra basal layer, we found that 50% of cases in group II and 14 % of cases in group IV showed mild staining, 36 % of group II had moderate staining and 14% of group IV had intense staining (Table XII to Table XIV).So the observation from this finding, supports the fact that there is a decrease in the Cx43 expression in OSCC with OSF than in OSF. It could be hypothesised that with malignant transformation of the OSF, there is a loss of Cx43 expression.

When we compared the intensity of expression of Cx43 between groups II (OSF) and Group IV (OSCC) in the connective tissue, there was no significant difference. Analysis of Cx43 staining intensity of the basal layer, supra basal layer and connective tissue, when compared between groups II and III and groupsIII and IV, was not found to be statistically significant(Table XV to XXII).

From the above findings, we state that Cx43 expression can be seen in the normal epithelia as stated by ***Trosko*** and ***Ruch***, such as those of skin, oesophagus, cervix, prostate, lung and CNS. Reduced expression or aberrant location of Cx43 protein or even loss of Cx43 expression has been reported in tumours arising from the epithelia of these tissues.[93]***Livny et al***reported that in normal oral mucosal cells, the expression of Cx43 was high when compared to that of oral cancer cells.[94]***Gitenay et al***stated that by restoring Cx43 expression, the cell growth rate and neoplastic ability of the tumours could be decreased significantly.[95]***Xia et al*** proposed that down-regulation of Cx43 expression was a common and a significant molecular event in carcinogenesis.[68]Given this fact, they hypothesize that Cx43 could be an important player in the early stage of development of OSCC. Cx43 expression in the fibroblasts of the connective tissue and its interaction with other cells could contribute additionally to fibrosis in OSF apart from exposure to arecanut alkaloids.[90]

The classical histopathological feature of OSF is considered to be deposition of collagen in the oral mucosa resulting from exposure to the areca-nut alkaloids.***Tilakaratne et al***stated that the fibrotic process in OSF is due to excessive collagen deposition and the disequilibrium in the extracellular matrix remodelling processes.[96] There are studies stating that there is a significant increase in the incidence of epithelial dysplasia, as the thickness of fibrosis

increased and advancement of fibrosis increases the risk of development of epithelial dysplasia in OSF.

Thus, the oral mucosal connective tissue changes in OSF influence the malignant transformation of the epithelium and its progression and metastasis. Thus, both the epithelial and connective tissue alterations work in a coordinated matter to result in epithelial-mesenchymal transition.[79]

` ***Pistorio et al*** studied the modulatory effects of Cx43 expression on GJIC with mast cells and fibroblasts. They used an in-vitro model which comprised of rodent derived peritoneal mast cell line (RMC-1 cells) and human dermal derived fibroblast. From their study, they stated that Cx43 is involved in promoting profibrotic fibroblast activities which can be modulated or regulated and can be considered as a potential therapeutic target in preventing excessive fibrosis.[90]

The limitations of this study are the small sample size and inability to distinguish the cell membrane and cytoplasmic staining to conclude that the downregulation of Cx43 could be an early event during oral carcinogenesis which could be considered as a biomarker for early changes in the malignant transformation of OSF. The observations of this study needs further research with a larger sample size.

Summary and Conclusion

- In this study, we had a total of 39 patients who comprised of 6 cases of group I (normal mucosa), 14 cases of group II (OSF), 12 cases of group III (epithelial dysplasia) and 7 cases of group IV (OSCC with history of OSF).
- Age distribution in group II (OSF) was 50% of the cases were within 25-50 years and 50% >50 years of age, whereas in group III (epithelial dysplasia) 50% of the cases were 25-50 years and 50% who were > 50 years of age. The distribution of age between the groups was found to be statistically significant.
- The study group II (OSF), group III (epithelial dysplasia) and group IV (OSCC with OSF) predominantly involved males. In group II (OSF), 71% were males and 29% were females. While in group III (epithelial dysplasia), 91% were males and 8% were females. In group IV (OSCC with OSF), 86% were males and 14% were females.
- In group II (OSF), 64% had the habit of areca nut chewing and 35% had the habit of chewing areca nut and tobacco. In group III (epithelial dysplasia), 58% had the habit of chewing tobacco, 8% had the habit of chewing areca nut and tobacco and 33.3% were smokers. In group IV (OSCC with OSF), 57% had the habit of smoking and areca nut chewing and 29% had the habit of smoking and tobacco and areca nut chewing.
- All the cases of group II (OSF) and group III (epithelial dysplasia) were taken from the buccal mucosa. In group IV (OSCC with OSF), 43% cases were from buccal mucosa, 14% from alveolar mucosa, 14% from palate and 29% from lateral border of tongue.

- Membranous expression of Cx43 was present in basal, suprabasal layers of epithelium and connective tissue. 93% of group II (OSF), 66% of group III(epithelial dysplasia) and 86% of group IV (OSCC with OSF) showed overall expression of Cx43.
- In the basal layer, group II (OSF), there was no expression of Cx43 in 71% of cases with 21% of the cases showing mild intensity pattern. 7.1% of cases showed moderate intensity of staining. In group IV (OSCC with OSF), all the cases did not express Cx43.
- In group I, group III and group IV, Cx43 was not expressed in the basal layer.
- There was a significant difference in the staining intensity of Cx43 in the suprabasal layer among the study groups.In Group II (OSF),50% of cases and 36% of cases showed mild and moderate intensity, respectively. 71% of cases in group IV (OSCC with OSF) did not express Cx43, 14.3 % of cases showed mild intensity and intense staining was seen in 14% of cases. In group III (epithelial dysplasia), 41.7% cases showed no expression, 33.3% cases showed mild intensity pattern and 25% cases showed moderate intensity.
- In Group IV, Cx43 expression was seen in two cases (28.6%) in the surface epithelium and the infiltrating epithelial islands in the connective tissue.
- On comparing the Cx43 staining intensity of connective tissue, in group II (OSF) 7% did not express Cx43, 36% had mild expression, 50% had moderate expression and 7% with intense expression. In group IV (OSCC with OSF), 71% showed mild intensity of staining, 14% cases showed

moderate intensity and 14% showed intense staining.In group III (epithelial dysplasia), 33% showed no expression, 42% cases showed mild intensity and 25% cases showed moderate intensity.

Cx43 was expressed in all the groups but its expression in the epithelium was not seen in four cases of OSF with dysplastic epithelium. Intense expression of Cx43 in the connective tissue was seen in OSF and OSCC. There was a reduced expression of Cx43 in cases of OSCC with OSF when compared to OSF cases. We postulate that downregulation of Cx43 expression could be an early event in carcinogenesis. These observations need to be further studied.

Bibliography

1. Gonzalez-Moles MA, Scully C, Ruiz-Avila I. Molecular findings in oral premalignant fields: update on their diagnostic and clinical implications. Oral diseases. 2012 Jan;18(1):40-7.
2. Max Parkin D, Bray F, Ferlay J, Pisani P, Global Cancer Statistics, 2002,(CA Cancer J Clin 2005;55:74 –108.) © American Cancer Society, Inc., 2005.
3. Reibel J, Gale N, Hille J, et al. Oral potentially malignant disorders and oral epithelial dysplasia. In: El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PPJ, eds. WHO Classification of Head and Neck Tumours. 4th ed. Lyon, France: IARC; 2017: 112-115.
4. Yang SF, Wang YH, Su NY, Yu HC, Wei CY, Hang Yu C, Chang YC, Changes in prevalence of precancerous oral submucous fibrosis from 1996 to 2013 in Taiwan: A nationwide population-based retrospective study, Journal of the Formosan Medical Association (2017),
5. Agents Classified by the IARC Monographs, Volumes 1–122
6. Paymaster JC. Cancer of the buccal mucosa—a clinical study of 650 cases in Indian patients. Cancer. 1956;9:431–435
7. Murti PR, Bhonsle RB, Pindborg JJ, Daftary DK, Gupta PC, Mehta FS. Malignant transformation rate in oral submucous fibrosis over a17-yr period. *Community Dent Oral Epidemol*. 1958;13:340-341
8. Wang YY, Tail YH, Wang WC, et al. Malignant transformation in 5071 southern Taiwanese patients with potentially malignant oral mucosal disorders. BMC Oral Health. 2014;14: 99.
9. Gupta PC, Mehta FS, Daftary DK, Pindborg JJ, Bhonsle RB, Jalnawalla PN, Sinor PN, Pitkar VK, Murti PR, Irani RR, Shah HT, Kadam PM, Iyer KS, Iyer HM, Hegde AK, Chandrashekar GK, Shiroff BC, Sahiar BE, Mehta

MN. Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. Community Dent Oral Epidemiol. 1980;8(6):283-333.

10. Jayasooriya PR, Jayasinghe KAN, Tilakaratne WM. Relationship between thickness of fibrosis and epithelial dysplasia in oral submucous fibrosis.Journal of Investigative and Clinical Dentistry (2011), 2, 171–175
11. Alexander D.B, Goldberg G.S. Transfer of Biologically Important Molecules Between Cells Through Gap Junction Channels. Current Medicinal Chemistry, 2003, 10, 2045-2058
12. Marc Chanson M, Watanabe M, O'Shaughnessy E M, Zoso A, Martin P.E. Connexin Communication Compartments and Wound Repair in Epithelial Tissue.Int J Mol Sci. 2018 May; 19(5): 1354.
13. Pindborg J.J, Sirsat S.M, Oral submucous fibrosis,O.S., O.M. & O.P. Volume 22, number 6, December 1966
14. Tang JG, Jian XF, Gao ML, Ling TY, Zhang KH, Epidemiological survey of oral submucous fibrosis in Xiangtan City, Hunan Province, China.,Community Dent Oral Epidemiol. 1997 Apr;25(2):177-80
15. Reichart P A, Nguyen X H, Betel quid chewing, oral cancer and other oral mucosal diseases in Vietnam: a review, J Oral Pathol Med, 37 (2008), pp. 511-514
16. Yang SF, Wang YH, Su NY, Yu HC, Wei CY, Hang Yu C, Chang YC, Changes in prevalence of precancerous oral submucous fibrosis from 1996 to 2013 in Taiwan: A nationwide population-based retrospective study, Journal of the Formosan Medical Association (2017).

17. Chiu CJ, Chang ML, Chiang CP, Hahn LJ, Hsieh LL, Chen CJ. Interaction of collagen-related genes and susceptibility to betel quid-induced oral submucous fibrosis. Cancer Epidemiol Biomarkers Prev. 2002 Jul;11(7):646-53.

18. Murti PR, Bhonsle RB, Pindborg JJ, Daftary DK, Gupta PC, Mehta FS, Malignant transformation rate in oral submucous fibrosis over a 17-year period, Community Dent Oral Epidemiol. 1985 Dec;13(6):340-1

19. Ranganathan K, Uma Devi M, Elizabeth J, Kirankumar K, Saraswathi TR, Oral submuocus fibrosis a case control study in chennai, south india; J Oral Path Med (2004) 33; 274 – 7

20. Lin KH, Lin CY, Liu CC, Chou MY, Lin JK, Arecoline N-Oxide: Its Mutagenicity and Possible Role as Ultimate Carcinogen in Areca Oral Carcinogenesis, Journal of Agriculture and Food Chemistry. 2011, 59, 3420–3428

21. Sundqvist K, Liu Y, Nair J, Bartsch H, Arvidson K, GrafströmRC.Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. Cancer Res. 1989 Oct 1;49(19):5294-8.

22. Scum A, Meghji S, Harvey W, Stimulation of Human Fibroblast Collagen Synthesis in Vitro by Y-Aminobutyric Acid, Biochemical Pharmacology, Vol. 36, No. 8, pp. 1333-1335, 1987

23. Utsunomiya H, Tilakaratne WM, Oshiro K, Maruyama S, Suzuki M, Ida-Yonemochi H, Cheng J, Saku T. Extracellular matrix remodeling in oral submucous fibrosis: its stage-specific modes revealed by immunohistochemistry and in situ hybridization. J Oral Pathol Med. 2005 Sep;34(8):498-507

24. Trivedy CR, Warnakulasuriya S, Peters TJ, Senkus R, Hazarey VK, Johnson NW, Raised tissue copper levels in oral submucous fibrosis, Journal of Oral pathology and medicine, 2000 Jul;29(6):241-8.

25. Haque MF, Harris M, Meghji S, Barrett AW. Immunolocalization of cytokines and growth factors in oral submucous fibrosis. Cytokine. 1998 Sep;10(9):713-9.

26. Tsai CH, Chou MY, Chang YC. The up-regulation of cyclooxygenase-2 expression in human buccal mucosal fibroblasts by arecoline: a possible role in the pathogenesis of oral submucous fibrosis. J Oral Pathol Med. 2003 Mar;32(3):146-53.

27. Massague J, TGF-β Signal Transduction, Annu. Rev. Biochem. 1998. 67:753–9

28. Deng YT, Chen H.M, Cheng S.J, Chiang C.P, Ping Kuo M.Y, Arecoline-stimulated connective tissue growth factor production in human buccal mucosal fibroblasts: Modulation by curcumin, Oral Oncology 45 (2009) e99–e105

29. Kamath V.V, Satelur K.P, Rajkumar K, Krishnamurthy K,Transforming growth factor beta 1 in oral submucous fibrosis: An Immunohistochemical study – Understanding the pathogenesis, Journal of Dental Research and Review, May-Aug 2014, Vol. 1, Issue 2 . 75

30. Moutasim KA, Jenei V, Sapienza K, Marsh D, Weinreb PH, Violette SM, Lewis MP, Marshall JF, Fortune F, Tilakaratne WM, Hart IR, Thomas GJ. Betel-derived alkaloid up-regulates keratinocyte alphavbeta6 integrin expression and promotes oral submucous fibrosis. J Pathol. 2011 Feb;223(3):366-77.x

31. Angadi PV, Kale AD, Hallikerimath S. Evaluation of myofibroblasts in oral submucous fibrosis: correlation with disease severity. J Oral Pathol Med. 2011 Mar;40(3):208-13.

32. Sirsat SM, Pindborg JJ, Subepithelial Changes in Oral Submucous Fibrosis,Actn path. et microbiol. sznndinav. 70, 161-173, 1967

33. Warnakulasuriya S, Johnson NW, van der Waal I, Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007 Nov;36(10):575-80

34. Ranganathan K, Kavitha R, Proliferation and apoptosis markers in oral submucous fibrosis. J Oral MaxillofacPathol. 2011 May-Aug; 15(2): 148–153.

35. Chaudhary M, Bajaj S, Bohra S , Swastika N, Hande A, The domino effect: Role of hypoxia in malignant transformation of oral submucous fibrosis;; Journal of Oral and Maxillofacial Pathology Vol. 19 Issue 2 May - Aug 2015 122

36. Chaturvedi P, Malik A, Nair D, Nair S, Mishra A, Garg A, Vaishampayan S .Oral squamous cell carcinoma associated with oral submucous fibrosis have better oncologic outcome than those without. Oral Surg Oral Med Oral Pathol Oral Radiol. 2017 Sep;124(3):225-230

37. Uikey A.K, Hazarey V.K, Vaidhya S.M, Estimation of serum antioxidant enzymes superoxide dismutase and glutathione peroxidase in oral submucous fibrosis: a biochemical study. Journal of Oral and Maxillofacial Pathology. 2003; 7: 44-45

38. Tilakaratne WM, Iqbal Z, Teh MT, Ariyawardana A, Pitiyage G, Cruchley A, Stewart,E. Hagi-Pavli JE, Lalli A, Waseem A, Parkinson EK, Fortune F,

Upregulation of HIF-1alpha in malignant transformation of oral submucous fibrosis; . J Oral Pathol Med 37: 372-377

39. Hassona Y, Cirillo N, Lim KP, Herman A, Mellone M, Thomas GJ, Pitiyage GN, Parkinson EK, Prime SS,Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF-β, Carcinogenesis. 2013 Jun;34(6):1286-95
40. Oshima R.G, Intermediate Filaments: A Historical Perspective, Exp Cell Res. 2007 June 10; 313(10): 1981–1994.
41. Heyden A, Huitfeldt H.S, Koppang H.S, Thrane P.S, Bryne M ,Brandtzaeg P, Cytokeratins as epithelial differentiation markers in premalignant and malignant oral lesions, J Oral Pathol Med Volume21, Issue1, January 1992, 7-11
42. Ranganathan K, Kavitha R, Sawant S.S, Vaidya M.M, Cytokeratin expression in oral submucous fibrosis - an immunohistochemical study, J Oral Pathol Med (2006) 35: 25–32
43. Teh MT, Tilakaratne WM, Chaplin T, Young BD, Ariyawardana A, Pitiyage G, Lalli A, Stewart JE, Hagi-Pavli E, Cruchley A, Waseem A, Fortune F. Fingerprinting genomic instability in oral submucous fibrosis. J Oral Pathol Med. 2008 Aug;37(7):430-6
44. Kalluri R, Weinberg R A. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009 Jun 1; 119(6): 1420–1428
45. Oliveira LR, Ribeiro-Silva A. Prognostic significance of immunohistochemical biomarkers in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2011 Mar;40(3):298-307.

46. Ekanayaka RP, Tilakaratne WM, Oral submucous fibrosis: review on mechanisms of malignant transformation. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016 Aug;122(2):192-9.

47. Alexander D.B, Goldberg G.S. Transfer of Biologically Important Molecules Between Cells Through Gap Junction Channels. Current Medicinal Chemistry, 2003, 10, 2045-2058

48. Loewenstein W.R, Kanno Y,Intercellular Communication And Tissue Growth,J Cell Biol. 1967 May 1; 33(2): 225–234

49. Musil LS, Goodenough DA. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. Cell. 1993;74:1065–1077

50. Delmar M, Coombs W, Sorgen P, Duffy HS, Taffet SM. Structural bases for the chemical regulation of Connexin43 channels. Cardiovasc Res. 2004;62:268–275

51. Solan JL, Lampe PD. Connexin 43 Phosphorylation – Structural Changes And Biological Effects. Biochem J. 2009 Apr 15; 419(2): 261–272.

52. Beyer EC, Paul DL, Goodenough DA. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. J Cell Biol. 1987 Dec; 105(6 Pt 1):2621-9.

53. Vinken M, Vanhaecke T, Papeleu P, Snykers S, Henkens T, Rogiers V. Connexins and their channels in cell growth and cell death. ell Signal. 2006 May;18(5):592-600. Epub 2005 Sep 23.

54. Zhou JZ, Jiang JX. Gap junction and hemichannel-independent actions of connexins on cell and tissue functions – An update.FEBS Lett. 2014 Apr 17; 588(8): 1186–1192.

55. Laird DW. Life cycle of connexins in health and disease. Biochem J. 2006;394:527–543.

56. Phillips SL, Williams CB, Joelle N, Zambrano JN ,Williams CJ, Yeh E, Connexin 43 in the development and progression of breast cancer: What's the connection? (Review),Int J Oncol. 2017 Oct; 51(4): 1005–1013.

57. Loewenstein W.R, Penn R.D Intercellular communication and tissue growth. II. Tissue regeneration, The Journal of Cell Biology, 33: 235-242, 1967.

58. Bonacquisti E, Nguyen J, Connexin 43 (Cx43) in Cancer: Implications for Therapeutic Approaches via Gap Junctions. Cancer Lett. 2019 Feb 1;442:439-444. doi: 10.1016/j.canlet.2018.10.043. Epub 2018 Nov 22.

59. King TJ, Fukushima LH, Hieber AD, Shimabukuro KA, Sakr WA, Bertram JS. Reduced levels of connexin43 in cervical dysplasia: inducible expression in a cervical carcinoma cell line decreases neoplastic potential with implications for tumor progression. Carcinogenesis. 2000 Jun;21(6):1097-109.

60. Dubina M.V, Iatckii N.A, Popov D.E, Vasil'ev S.V, Krutovskikh V.A,Connexin 43, but not connexin 32, is mutated at advanced stages of human sporadic colon cancer, Oncogene (2002) 21, 4992 – 4996

61. Kańczuga-Koda L, Sulkowska M, Koda M, Reszeć J, Famulski W, Baltaziak M, Sulkowski S,Expression of connexin 43 in breast cancer in comparison with mammary dysplasia and the normal mammary gland. Folia Morphol (Warsz) 2003;62:439–442

62. Tanaka T, Kimura M, Ishiguro H, Mizoguchi K, Takeyama H. Connexin 43 expression is associated with poor survival in patients with esophageal

squamous cell carcinoma.MolClinOncol. 2016 Jun;4(6):989-993. Epub 2016 Mar 22.

63. Jia Y, Xu CX, Yang WB. Expressions of connexin 32 and connexin 43 in patients with gastric precancerous lesion after eradication of Helicobacter pylori.ZhongNan Da XueXueBaoYiXueBan. 2008 Jul;33(7):628-33.
64. Tang B, Peng ZH, Yu PW, Yu G, Qian F. Expression and significance of Cx43 and E-cadherin in gastric cancer and metastatic lymph nodes.Med Oncol (2011) 28:502–508
65. Poyet C, Buser L, Roudnicky F, Detmar M, Hermanns T, Mannhard D, Höhn A, Rüschoff J, Zhong Q, Sulser T, Moch H, Wild PJ.Connexin 43 expression predicts poor progression-free survival in patients with non-muscle invasive urothelial bladder cancer.J ClinPathol. 2015 Oct;68(10):819-2
66. Erdem H, AkçayÇelik M. Evaluation of the Relationship between Cervical Intraepithelial Neoplasia Grades and Connexin 43.Middle Black Sea Journal of Health Science December 2017; 3(3):15-19
67. Hagemann IS, Pasha TL, Roberts SA, Yum SW, Zhang PJ. Aberrant connexin 43 and 26 expression in cervical dysplasia. Anal Quant CytolHistol. 2012 Feb;34(1):28-40
68. Xia J, Liu X, Tao X, Hong Y, Chen X, Dai Y, Huang Y, Cheng B.Expression of gap junctional protein connexin43 during 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis.Journal of Molecular Histology [01 Jun 2009, 40(3):183-188]
69. Feng Y, Kang XJ, Li CH, Nie MH. Expression of connexin 43 in tongue carcinogenesis,Chinese journal of medical genetics. 2013 Apr;30(2):237-9.

70. Feng Y, Kang XJ, Li CH, Nie MH, Expression of cytokeratin 19 and connexin 43 in 4-nitroquinoline-l-oxide-induced rat tongue carcinogenesis, West China journal of stomatology 31(3):237-41 · June 2013

71. Brockmeyer P, Hemmerlein B, Jung K, Fialka F, Brodmann T, Matthias Gruber R, Schliephake H, Josef Kramer F, Connexin subtype expression during oral carcinogenesis: A pilot study in patients with oral squamous cell carcinomaMolClinOncol. 2016 Feb; 4(2): 298–302.

72. Brockmeyer P, Jung K, Perske C, Schliephake H, Hemmerlein B, Membrane connexin 43 acts as an independent prognostic marker in oral squamous cell [carcinoma. International Journal Of Oncology 45: 273-281, 2014

73. Puzzo L, Caltabiano R, Parenti R, Trapasso S, Allegra E. Connexin 43 (Cx43) Expression in Laryngeal Squamous Cell Carcinomas: Preliminary Data on Its Possible Prognostic Role. Head Neck Pathol. 2016 Sep; 10(3): 292–29.

74. Globocan. Estimated cancer incidence, mortality and prevalence worldwide in 2012.

75. Montero PH, Patel SG. Cancer of the oral cavity. Surgical Oncology Clinics. 2015 Jul 1;24(3):491-508.

76. Le Campion AC, Ribeiro CM, Luiz RR, da Silva Júnior FF, Barros HC, dos Santos KD, Ferreira SJ, Gonçalves LS, Ferreira SM. Low survival rates of oral and oropharyngeal squamous cell carcinoma. International journal of dentistry. 2017;2017.

77. Petti S. Pooled estimate of world leukoplakia prevalence: a systematic review. Oral oncology. 2003 Dec 1;39(8):770-80.

78. Silvermann S, Gorsky M, Lozada F. Oral leukoplakia and malignant transformation. Cancer. 1984;35:563-8

79. Ray JG, Ranganathan K, Chattopadhyay A. Malignant transformation of oral submucous fibrosis: overview of histopathological aspects. Oral surgery, oral medicine, oral pathology and oral radiology. 2016 Aug 1;122(2):200-9.

80. Mehrotra R, Yadav S. Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations. Indian journal of cancer. 2006 Apr 1;43(2):60.

81. Sarode SC, Sarode GS. Better grade of tumor differentiation of oral squamous cell carcinoma arising in background of oral submucous fibrosis. Medical hypotheses. 2013 Oct 1;81(4):540-3.

82. Balaji P, Govindraju P, Gupta A, Pawar Y, Gazge NM. Oral Squamous Cell Carcinoma in Background of Oral Submucous Fibrosis: A Case Report. IJSS. 2015 May;1(12):40.

83. Hazarey VK, Erlewad DM, Mundhe KA, Ughade SN. Oral submucous fibrosis: study of 1000 cases from central India. Journal of oral pathology & medicine. 2007 Jan;36(1):12-7.

84. Sinor PN, Gupta PC, Murti PR, Bhonsle RB, Daftary DK, Mehta FS, Pindborg JJ. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. Journal of oral pathology & medicine. 1990 Feb;19(2):94-8.

85. Kanodia S, Giri VP, Giri OP, Devi MP, Garima Y. Assessment of anxiety, depression, and serum cortisol level in oral submucous fibrosis patients: A controlled clinical trial. European journal of dentistry. 2017 Jul;11(3):293.

86. Fernandez-Cobo M, Gingalewski C, Drujan D, De Maio A. Downregulation of connexin 43 gene expression in rat heart during inflammation. The role of tumour necrosis factor. Cytokine. 1999 Mar 1;11(3):216-24.

87. Aasen T, Graham SV, Edward M, Hodgins MB. Reduced expression of multiple gap junction proteins is a feature of cervical dysplasia. Molecular cancer. 2005 Dec;4(1):31.

88. Y Han, RJ Zhang, Y Chen, SW Yum, Pasha T, EE Furth. Connexin43 Expression Increases in the Epithelium andStroma along the Colonic Neoplastic Progression Pathway: Implications for Its Oncogenic Role.Gastroenterology Research and Practic. Volume 2011, Article ID 561719, 8 pages

89. Meşe G, Richard G, White TW. Gap junctions: basic structure and function. Journal of Investigative Dermatology. 2007 Nov 1;127(11):2516-24.

90. Pistorio AL, Ehrlich HP. Modulatory effects of connexin-43 expression on gap junction intercellular communications with mast cells and fibroblasts. Journal of cellular biochemistry. 2011 May;112(5):1441-9.

91. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. The Journal of clinical investigation. 2009 Jun 1;119(6):1429-37.

92. Jamieson S, Going JJ, D'Arcy R, George WD. Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. J Pathol. 1998 Jan;184(1):37-43.

93. Trosko JE, Ruch RJ. Cell-cell communication in carcinogenesis. Front Biosci. 1998 Feb 15;3(3):d208-236.

94. Livny O, Kaplan I, Reifen R, Polak-Charcon S, Madar Z, Schwartz B. Lycopene inhibits proliferation and enhances gap-junction communication of

KB-1 human oral tumor cells. The Journal of nutrition. 2002 Dec 1;132(12):3754-9.

95. Gitenay D, Lyan B, Talvas J, Mazur A, Georgé S, Caris-Veyrat C, Rock E. Serum from rats fed red or yellow tomatoes induces Connexin43 expression independently from lycopene in a prostate cancer cell line. Biochemical and biophysical research communications. 2007 Dec 21;364(3):578-82.

96. Tilakaratne WM, Iqbal Z, Teh MT, Ariyawardana A, Pitiyage G, Cruchley A, Stewart JE, Hagi-Pavli E, Lalli A, Waseem A, Parkinson EK, Fortune F. Upregulation of HIF-1alpha in malignant transformation of oral submucous fibrosis. J Oral Pathol Med. 37 (2008) 372-7.

Annexures

ANNEXURE I

INSTITUTIONAL ETHICSCOMMITTEE FORM

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003-06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOMSOEVER IT MAY CONCERN

Date : 06.01.2018
Place : Chennai

From
The Institutional Review Board,
Ragas Dental College and Hospital,
Uthandi, Chennai - 600119.

The dissertation topic titled **"EXPRESSION OF CONNEXIN-43 IN ORAL SUBMUCOUS FIBROSIS AND ITS MALIGNANT TRANSFORMATION TO ORAL SQUAMOUS CELL CARCINOMA"** submitted by **Dr.R.N.MUGUNDAN,** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.

Dr. N. S. AZHAGARASAN, M.D.S.,
Member Secretary,
Institutional Ethics Board,
Ragas Dental College and Hospital,
Uthandi, Chennai - 600119.

RAGAS DENTAL COLLEGE Chennai

ANNEXURE II

DISSERTATION PROTOCOL

Title: Expression of Connexin 43 (Cx43) in oral submucous fibrosis and in its malignant transformation

Name and designation of the principal investigator:-

Dr. R.N.Mugundan,

I year post graduate student

Department of Oral and Maxillofacial Pathology

Name of HOD & staff in charge:-

Dr .K .Ranganathan, MDS, MS (Ohio), PhD

Dr .K .Umadevi, MDS

Dr . Elizabeth Joshua, MDS

Dr .T .Rooban, MDS

Department where project is to be carried out:-

Department of Oral and Maxillofacial Pathology,

Ragas Dental College,

Chennai

Duration of the project:I year

Background :-

Head and neck cancers are the 11th most common malignancy worldwide. Oral squamous cell carcinoma (OSCC) can occur in various parts of the oral cavity like the buccal mucosa, gingiva, tongue and the palate. The tumor has a high mortality as a result of its high invasiveness and metastatic capacity. The OSCCs are usually preceded by premalignant lesions which are now referred to as Oral Potentially Malignant Disorders (OPMD) by World Health Organization (WHO). Since not all of the OPMDs progressed to malignancy. .Features of epithelial

dysplasia are seen in advanced OSF and could lead to their malignant transformation. Cellular cohesion is maintained by Gap junctions that connects the cytoplasm of one adjacent cell to the other, Connexins are gap junction proteins that are expressed throughput the epithelium. Among them Connexin 43 (Cx43) is seen expressed extensively thoughout the tissues. Various studies have described the reduced expression of Cx43 in premalignant lesions and in carcinomas. There are no studies in the literature that had analyzed the Cx43 in oral submucous fibrosis and in its malignant transformation to oral squamous cell carcinoma

Hypothesis:-

There is no difference in the expression of Cx43 among OSF, Epithelial dysplasia and cases where OSF had undergone malignant transformation.

Aim:-

To evaluate the expression of Connexin 43 (Cx43), in formalin fixed paraffin embedded tissues of oral submucous fibrosis, epithelial dysplasia and OSF that had undergone malignant transformation to oral squamous cell carcinoma

Objectives:-

- To study the expression of Cx43in formalin fixed paraffin embedded tissues of Oral submucous fibrosis byIHC
- To study the expression of Cx43in formalin fixed paraffin embedded tissues of epithelial dysplasia by IHC
- To study the expression of Cx43 in in formalin fixed paraffin embedded tissues of OSF with OSCC byIHC.
- To study the expression of Cx43 in formalin fixed paraffin embedded normal epithelial tissue by IHC
- To compare the expression of Cx43 among the study groups

Materials and Methods:-

<u>Study groups</u>:-

- Group - I:Normal oral mucosa obtained with the patients consent during third molar extraction(n=6)

- Group - II: Clinically and histopathologically diagnosed cases of oral submucous fibrosis (n=14)
- Group- III: Clinically and Histopathologically diagnosed cases of Epithelial Dysplasia (n=12)
- Group-IV: Clinically and histopathologically diagnosed cases of OSCC with the history of OSF (n=7)

Selection criteria:-

- Group I–Normal mucosa obtained during removal of impacted third molar in the outpatient department of Oral and Maxillofacial Surgery department.
- Group II–The criteria for selection of OSF patients were difficulty in opening the mouth, burning sensation of the mouth, palpable vertical fibrous bands in the oral mucosa. Histopathological criteria for OSF cases were epithelial atrophy, increased deposition of collagen in the oral mucosa,reduced vascularity, reduced number of inflammatory cells, and sub-epithelial hyalinization. The OSF was histopathologically graded by criteria given by Sirsat and Pindborg.
- Group III - Clinically and histopathologically confirmed cases of Epithelial dysplasia which were graded histologically as mild, moderate and severe
- Group IV- Clinically and histopathologically confirmed cases of OSCC with a history of OSF.

Institutional review board:-

This study was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai.(Annexure I)

Study setting:-

Department of Oral and maxillofacial pathology, Ragas Dental College and Hospital.

Methodology:-

- Archival tissue samples of oral submucous fibrosis, epithelial dysplasia and oral squamous cell carcinoma accessed from Department of Oral Pathology, Ragas Dental College and Hospital.

- Demographic and pertinent details such as patient's age, gender, past medical and dental history, history of drugs and trauma were obtained from records for OSF cases, OSCC arising in OSF cases and normal cases.
- In group I, incisional biopsy were taken when patients were undergoing minor surgery for extraction of impacted teeth for normal oral mucosa after obtaining patient consent.
- The tissue biopsied were rinsed and immediately transferred to 10% buffered formalin.
- After adequate fixation, tissues were embedded in paraffin.
- From the paraffin embedded blocks 5 micron thick sections were cut and used for routine hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining.
- Positive control for Cx43 was goat heart tissue sections.

Immunohistochemistry was performed by:-

Antibodies used:-

Primary antibody –Anti-Cx43 rabbit monoclonal antibody, Dilution: 1:100 CAT NO- 71-0700 (*ThermoFisherScientific*^{TM})

Secondary antibody-Poly Excel-HRP Micro polymer IHCDetection system, Pierce IHC Detection Kit, CAT NO- 36000(*ThermoFisherScientific*^{TM})

Procedure:-

The slides were dewaxed in xylene and rehydrated through grades of alcohol to water. The sections on the slides were flooded with Harris's hematoxylin for 5 minutes. The slides were washed in running tap water for 5 minutes. The slides were differentiated in 1% acid alcohol for 5 minutes. The slides were washed well in running tap water for 5 minutes. The tissue sections on the slides were then stained in eosin for 30 seconds. The slides were washed in running tap water for 1 minute. The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope.

Evaluation of H&E sections:-

- The H&E stained sections were thoroughly examined. Oral squamous cell carcinomas were graded as well differentiated, moderately differentiated and poorly differentiated.
- Epithelial dysplasia were graded as mild, moderate and severe.
- Oral submucous fibrosis was graded histologically by criteria given by Sirsat and Pindborg.
- Corresponding sections as examined by H & E were stained by IHC to detect Cx43 expression.
- The positive control was a section of goat heart tissue that was previously shown to be positive for Cx43.
- Cx43 expression was evaluated as brown membranous staining in the epithelial cells.
- The cells were then assessed for staining intensity.

Intensity of staining:-

The staining intensity was analysed in the study groups. Each case was graded as (-) nil or absence of stain, (+) mild, (++) moderate and (+++) intensively stained based on the intensity of staining taken up by the tissue as observed by two blinded observers independently with respect to positive control.

Tissue localization:-

Epithelial cells that exhibited brown membranous staining were counted as positive for expression of Cx43. The sections were initially scanned at low power. For sections that showed heterogeneous staining, the predominant pattern was taken into account for scoring.

Cellular localization of stain:-

Brown Cx43 staining was seen as cytoplasmic or membranous granules. The stained slides were screened, examined systematically for Cx43 expression in cytoplasmic portion and walls of the epithelial cell.

Statistical test used: The staining intensity between each groups was evaluated using Chi-squared test.Data analysis to be done using *SPSS software (Statistical Package of Social Science) version 21*.

Signature of principal investigator

Signature of Head of Department

Remarks of committee

Permission Granted **YES / NO**

Modifications / comments

ANNEXURE III

CASE SHEET

Ragas Dental College & Hospital –Chennai

Date of Diagnosis:

Type of Biopsy:

Name : Age : Gender:

Marital Status : Occupation :

Religion : Education:

Dietary Habits : Vegetarian/Mixed/Non-Vegetarian

Spice : Spicy/Moderate/Non Spicy

CHEWING HABIT : (Swallowing/spitting/Pouching - R/L side)

	Method	Frequency/ Day	Quantity/ Day	Duration Month/Year

SMOKING HABIT :

TYPE	Number of Days	Duration- Months/Year	Ex Smoker Duration
Cigarette with filter without filter/beedi/pipe/others			

Any other Significant History Of alcohol and Drug abuse :

Medical And Family History :

Dental History :

Presenting lesion :

Mouth Opening :

Tongue Protrusion :

Cheek Flexibility :

SITE OF THE LESION:

	LEFT	RIGHT
1 .Gingiva		
2.BuccalMucosa		
3 . Labial Mucosa		
4 . Vestibule –Buccal		
5.Tongue - Dorsal Surface - Ventral surface		
8. Palate - Hard Palate - Soft palate		
-		
9. Uvula		
10. Floor of the mouth		
11.Tonsillar area		
12. Oropharynx		

Description of Lesion :

Provisional Diagnosis :'

Histopathological Diagnosis:

ORAL SUBMUCOUS FIBROSIS	**STAGE**
STAGE – 1	
STAGE - 2	
STAGE – 3	
STAGE - 4	

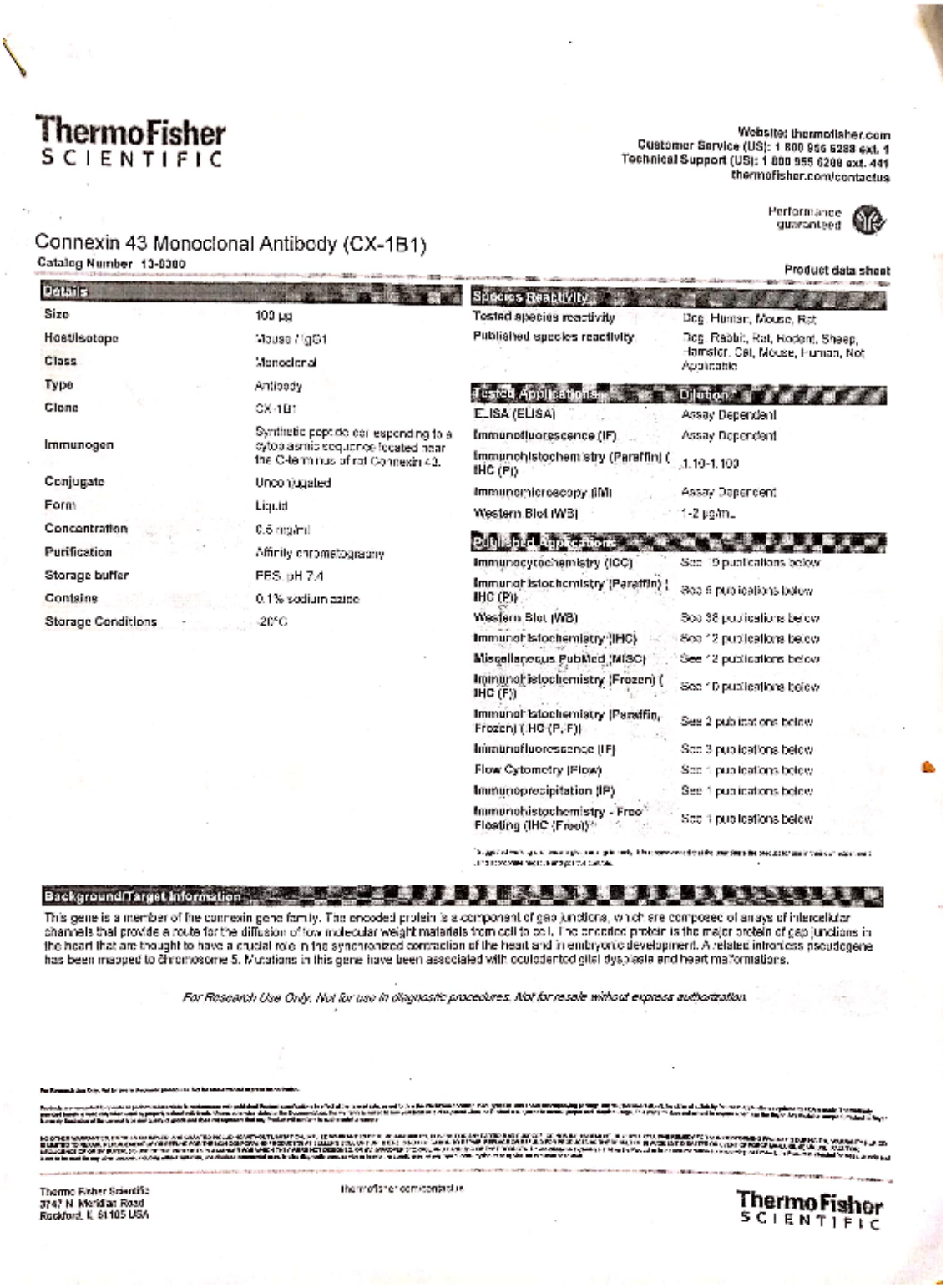
MILD DYSPLASIA	**MODERATE DYSPLASIA**	**SEVERE DYSPLASIA**

WELL DIFFRENTIATED OSCC	**MODERATELY DIFFRENTIATED OSCC**	**POORLY DIFFRENTIATED OSCC**

Treatment Done:

ANNEXURE IV

Connexin 43 – Cx43 Antibody FACT SHEET



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Connexin 43 Monoclonal Antibody (CX-1B1)

Catalog Number 13-8300

Product data sheet

Details	
Size	100 µg
Host/Isotype	Mouse / IgG1
Class	Monoclonal
Type	Antibody
Clone	CX-1B1
Immunogen	Synthetic peptide corresponding to a cytoplasmic sequence located near the C-terminus of rat Connexin 43.
Conjugate	Unconjugated
Form	Liquid
Concentration	0.5 mg/ml
Purification	Affinity chromatography
Storage buffer	PBS, pH 7.4
Contains	0.1% sodium azide
Storage Conditions	-20°C

Species Reactivity	
Tested species reactivity	Dog, Human, Mouse, Rat
Published species reactivity	Dog, Rabbit, Rat, Rodent, Sheep, Hamster, Cat, Mouse, Human, Not Applicable

Tested Applications	Dilution
ELISA (ELISA)	Assay Dependent
Immunofluorescence (IF)	Assay Dependent
Immunohistochemistry (Paraffin) (IHC (P))	1:10-1:100
Immunomicroscopy (IMI)	Assay Dependent
Western Blot (WB)	1-2 µg/m

Published Applications	
Immunocytochemistry (ICC)	See 9 publications below
Immunohistochemistry (Paraffin) (IHC (P))	See 6 publications below
Western Blot (WB)	See 38 publications below
Immunohistochemistry (IHC)	See 2 publications below
Miscellaneous Published (MISC)	See 2 publications below
Immunohistochemistry (Frozen) (IHC (F))	See 1 publications below
Immunohistochemistry (Paraffin, Frozen) (IHC (P, F))	See 2 publications below
Immunofluorescence (IF)	See 3 publications below
Flow Cytometry (Flow)	See 1 publications below
Immunoprecipitation (IP)	See 1 publications below
Immunohistochemistry - Free Floating (IHC (Free))	See 1 publications below

Background/Target Information

This gene is a member of the connexin gene family. The encoded protein is a component of gap junctions, which are composed of arrays of intercellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell. The encoded protein is the major protein of gap junctions in the heart that are thought to have a crucial role in the synchronized contraction of the heart and in embryonic development. A related intronless pseudogene has been mapped to chromosome 5. Mutations in this gene have been associated with oculodentodigital dysplasia and heart malformations.

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Product Images For Connexin 43 Monoclonal Antibody (CX-1B1)

Connexin 43 Antibody (13-8300) in IHC (P)

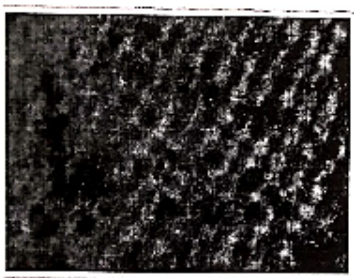
Immunohistochemistry analysis of Connexin 43 showing staining in the membrane of paraffin-embedded human lung adenocarcinoma (right) compared to a negative control without primary antibody (left). To expose target proteins, antigen retrieval was performed using 10mM sodium citrate (pH 6.0), microwaved for 8-15 min. Following antigen retrieval, tissues were blocked in 3% H2O2-methanol for 15 min at room temperature, washed with ddH2O and PBS, and then probed with a Connexin 43 monoclonal antibody (138300) diluted in 3% BSA-PBS at a dilution of 1:20 overnight at 4°C in a humidified chamber. Tissues were washed extensively in PBST and detection was performed using an HRP-conjugated secondary antibody followed by colorimetric detection using a DAB kit. Tissues were counterstained with hematoxylin and dehydrated with ethanol and xylene to prep for mounting.

Connexin 43 Antibody (13-8300) in IHC (P)

Immunohistochemistry analysis of Connexin 43 showing staining in the membrane of paraffin-embedded mouse heart tissue (right) compared to a negative control without primary antibody (left). To expose target proteins, antigen retrieval was performed using 10mM sodium citrate (pH 6.0), microwaved for 8-15 min. Following antigen retrieval, tissues were blocked in 3% H2O2-methanol for 15 min at room temperature, washed with ddH2O and PBS, and then probed with a Connexin 43 monoclonal antibody (138300) diluted in 3% BSA-PBS at a dilution of 1:100 overnight at 4°C in a humidified chamber. Tissues were washed extensively in PBST and detection was performed using an HRP-conjugated secondary antibody followed by colorimetric detection using a DAB kit. Tissues were counterstained with hematoxylin and dehydrated with ethanol and xylene to prep for mounting.

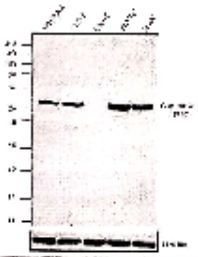
Connexin 43 Antibody (13-8300) in IHC (P)

Immunohistochemistry analysis of Connexin 43 showing staining in the membrane of paraffin-embedded mouse testis tissue (right) compared to a negative control without primary antibody (left). To expose target proteins, antigen retrieval was performed using 10mM sodium citrate (pH 6.0), microwaved for 8-15 min. Following antigen retrieval, tissues were blocked in 3% H2O2-methanol for 15 min at room temperature, washed with ddH2O and PBS, and then probed with a Connexin 43 monoclonal antibody (138300) diluted in 3% BSA-PBS at a dilution of 1:20 overnight at 4°C in a humidified chamber. Tissues were washed extensively in PBST and detection was performed using an HRP-conjugated secondary antibody followed by colorimetric detection using a DAB kit. Tissues were counterstained with hematoxylin and dehydrated with ethanol and xylene to prep for mounting.



Connexin 43 Antibody (13-8300) in IHC

Immunofluorescence analysis of Connexin43 (Cx43) was performed on sections of adult brain. Tissue sections on slides were probed for 24 h at 4°C in a humidified chamber with mouse monoclonal anti-Cx43 (Cat. No. 13-8300) at an antibody concentration of 1-2 µg/ml diluted in 50 mM Tris-HCl, pH 7.4, containing 1.5% NaCl, 0.3% Triton X-100 (TBST) and 4% normal goat serum. After overnight incubation, sections were washed extensively for 1 h in TBST, and detection of primary antibody was performed for 1.5 h at room temperature with AlexaFluor-488-conjugated donkey anti-mouse diluted 1:600 in TBST. Sections were then washed in TBST, then in TBS (without Triton) and then coverslipped with anti-fade medium. Images were taken on a Zeiss Z2 scanning microscope at x40 objective magnification, and show immunofluorescence labelling of Cx43 localized at gap junctions between astrocytes in the thalamus of adult mouse brain. Data courtesy of Dr. James Nagy's lab.



Connexin 43 Antibody (13-8300) in WB

Western blot analysis was performed on whole cell extracts (30 ug lysate) of NTERA-2 (Lane 1),C2C12 (Lane 2), Caco-2 (Lane 3),HUVEC (Lane 4) and HAFC (Lane 5). The blots were probed with Anti-Connexin 43 Mouse Monoclonal Antibody (Product# 138300, 1-2 ug/mL) and detected by chemiluminescence using Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate (Product # 626520, 1:4000 dilution). A ~ 50 kDa band corresponding to Connexin 43 was observed across cell lines tested except Caco-2. Known quantity of protein samples were electrophoresed using Novex® NuPAGE® 12 % Bis-Tris gel (Product # NP0342BOX), XCell SureLock™ Electrophoresis System (Product # EI0002) and Novex® Sharp Pre-Stained Protein Standard (Product # LC5800). Resolved proteins were then transferred onto a nitrocellulose membrane with iBlot® 2 Dry Blotting System (Product # IB21001). The membrane was probed with the relevant primary and secondary Antibody following blocking with 5 % skimmed milk. Chemiluminescent detection was performed using Novex® ECL Chemiluminescent Substrate Reagent Kit (Product # WP20005).

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ANNEXURE – V

PLAGIARISM REPORT

URKUND

Urkund Analysis Result

Analysed Document:	mukundan whole thesis.doc (D47677126)
Submitted:	2/7/2019 7:59:00 AM
Submitted By:	mukundalonso27@gmail.com
Significance:	2 %

Sources included in the report:

Main dissertation.docx (D45722251)
thesis.docx (D35101763)
manuscript.docx (D46440537)
http://www.cancerjournal.net/article.asp?issn=0973-1482;year=2018;volume=14;issue=3;spage=597;epage=603;aulast=Biradar;type=3

Instances where selected sources appear:

4

ANNEXURE VI

DEPARTMENT DECLARATION FORM

The study title "Expression of Connexin 43 (Cx43) in oral submucous fibrosis and in its malignant transformation" have been done under the guidance of the staffs of the Department of Oral Pathology and Microbiology during my post - graduation during 2016 - 2019. The same has been submitted as a part of the syllabus MDS degree programme in Oral Pathology and Microbiology of the Tamil Nadu Dr. M .G.R . Medical University, Chennai.

I shall publish in full or part of this work in any media with only with the prior written approval of the head of the department.

R.N. Mugundan

Post-graduate student 2016-2019

Department of Oral and Maxillofacial Pathology

Ragas Dental College and Hospital, Chennai

ANNEXURE VII

ABBREVIATIONS

1. OSCC	-	Oral Squamous Cell Carcinoma
2. OPMD	-	Oral Potentially Malignant Disorder
3. WHO	-	World Health Organization
4. OSF	-	Oral Submucous Fibrosis
5. IARC	-	International Association of Research on Cancer
6. TGF-β	-	Transforming Growth Factor –β
7. NF-κB	-	Nuclear Factor – kappa B
8. IL-6	-	Interleukin 6
9. PG-E2	-	Prostaglandin E2
10. GJIC	-	Gap Junctional Intercellular Communication
11. GJ	-	Gap Junctions
12. Cx43	-	Connexin 43
13. IHC		Immunohistochemistry
14. H&E		Hematoxylin& Eosin
15. PBS		Phosphate Buffered Saline
16. HRP		Horse Radish Peroxidase
17. DAB		Diamino benzene
18. DPX		Dibutylphthalate Polystyrene Xylene

19. CTGF	-	Connective Tissue Growth Factor
20. MMP	-	Matrix Metalloproteinases
21. TIMP	-	Tissue Inhibitors of Matrix Metalloproteinases
22. TNF- α	-	Tumor Necrosis Factor **α**
23. VEGF	-	Vascular Endothelial Growth Factor
24. IFN- γ	-	Interferon **γ**
25. IL-1	-	Interleukin – 1
26. FGF	-	Fibroblast Growth Factor
27. PBMC	-	Peripheral Blood Mononuclear cells
28. COX-2	-	CycloOxygenase 2
29. TGF-β1	-	Transforming Growth Factor –β1
30. TGF-β2	-	Transforming Growth Factor –β2
31. αVβ6	-	Alpha V beta-6 Integrin
32. ECM	-	Extra cellular Matrix
33. SASP	-	Senescent Associated Secretory Phenotype
34. MVD	-	Mean Vascular Diameter
35. CK	-	Cytokeratin
36. HMWCK	-	High Molecular Weight Cytokeratin
37. PAN-CK	-	Pan Cytokeratin
38. SNP	-	Single Nucleotide Polymorphism
39. LOH	-	Loss Of Heterozygosity
40. EMT		Epithelial Mesenchymal Transition
41. ZO-1		ZonulaOccludens– 1

42. αSMA		Alpha Smooth Muscle Actin
43. FSP-1		Fibroblast Specific Protein – 1
44. kD		Kilo Dalton
45. rER		Rough Endoplasmic Reticulum
46. CDK		Cyclin Dependent Kinase
47. CIP		Cyclin Inhibitory Proteases
48. CSG		Chronic Superficial Gastritis
49. Cx32	-	Connexin 32
50. HP	-	Helicobacter Pylori
51. SIL		Squamous Intraepithelial Neoplasia
52. HSIL	-	High Grade Squamous Intraepithelial Neoplasia
53. 4NQO	-	4-Nitroquinolone-1-Oxide
54. RMC-1		Rodent derived peritoneal Mast Cell – 1